THE BIOCHEMICAL JOURNAL

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The Enzymic Hydrolysis of β-Glucosides

2121
884/12

By E. M. CROOK and B. A. STONE
(*Biochemistry Department, University College, London*)

(*Received* 11 *April* 1956)

The present work arose out of an attempt to understand the course of cellulose breakdown in preparations of cellulolytic enzymes which also contained cellobiase activity. Previous investigations of the β-glucosidases have been of three kinds—an examination of their specificity ranges (reviewed by Veibel, 1950), the demonstration of their reversibility and the study of glucosyl transfer. Croft-Hill (1898), in demonstrating the reversibility of enzyme reactions, showed the formation of disaccharides from glucose in the presence of yeast enzymes. Gentiobiose was later found to be the chief product of this type of reaction catalysed by β-glucosidases (Bourquelot & Bridel, 1919; Bourquelot, Hérissey & Coirre, 1913), and it is now a recognized method of preparing this sugar (Helferich & Leete, 1942). More recently, Peat, Whelan & Hinson (1952) have shown that five β-linked glucose disaccharides can be obtained by incubating 60% glucose with almond emulsin over a period of 5 weeks. Aliphatic and aromatic alcohols, too, can condense with glucose in the presence of β-glucosidases (Bourquelot & Bridel, 1913; Veibel, 1936; Vintilescu, Ionescu & Kizyk, 1934, 1935*a*, *b*; Vintilescu, Ionescu & Solomon, 1935).

The first to describe transglycosylation and to realize the complementary role of the reactants was Rabaté (1935), who showed that methyl β-glucoside was formed when piceoside (*p*-hydroxyacetophenone β-glucoside) was incubated with methanol and extracts of *Salix purpurea*, and that glucosidically linked glucose was necessary for the reaction. Miwa, Takano, Mafune & Furutani (1949), Miwa & Takano (1950), Takano & Miwa (1950) and Jermyn & Thomas (1953) confirmed these results and extended them, using a series of fungal and plant extracts acting upon several aromatic β-glucosides in the presence of methanol, ethanol or butanol. In 1953 Crook & Stone reported the formation of a series of oligosaccharides from cellobiose in the presence of various enzyme preparations. Since then Barker, Bourne & Stacey (1953), Barker, Bourne, Hewitt & Stacey (1955), Buston & Jabbar (1954), Giri, Nigam & Srinivasan (1954) and Kooiman, Roelofsen & Sweeris (1954) have described similar reactions with various mould enzyme preparations. Manners (1955) has observed the formation of higher saccharides during the action of a barley β-glucosidase preparation on cellobiose. The present paper is a fuller account of the work previously reported (Crook & Stone, 1953) and an attempt to elucidate some of the reactions occurring. Its substance has already formed part of a thesis (Stone, 1954).

EXPERIMENTAL

Materials

Substrates. Salicin and cellobiose were products of L. Light and Co., Colnbrook, Bucks. Methyl β-glucoside was prepared by the method of Koenigs & Knorr (1901). The acetobromoglucose required for this preparation was obtained according to Bárczai-Martos & Körösy (1950), and the product was recrystallized from ethyl acetate, m.p. 110·0–110·5°. Laminaribiose was a gift of Dr W. J. Whelan. Gentiobiose was extracted from gentian root (*Gentiana lutea* L.) according to the method of Haworth & Wylam (1923). From gentian root (400 g.) was obtained 20 g. of a brown gum which was dissolved in 75 ml. of water and fractionated on a charcoal column (3·2 cm. × 33·5 cm.; 77 g. of Speakman no. 130, sieved charcoal). Gentiobiose was obtained from the pooled 5% and 7·5% ethanol fractions by preparative paper electrophoresis.

Oligosaccharides from laminarin. Laminarin (2·5 g.) from *Laminaria cloustoni*, a gift of Professor T. Dillon, was heated with 240 ml. of N oxalic acid at 95° for 5 hr. This time was calculated from the data of Connell, Hirst & Percival (1950) to give the maximum yield of oligosaccharides of 3–5 glucose units. The acid was neutralized with $CaCO_3$ and the calcium oxalate filtered off. The filtrate was heated at 90° for 1 hr., refiltered and freeze-dried to a white powder. When chromatographed in *n*-butanol–pyridine–water (6:4:3, by vol.), this material showed a series of spots, the lowest of which coincided with glucose and laminaribiose markers, and which gave a straight line on plotting log R_G against the spot number up to seven as required for a homologous series according to French & Knapp (1950). It may thus be assumed that a negligible amount of transglycosylation occurred during hydrolysis and that the material consisted of a series of oligosaccharides ranging from glucose through laminaribiose and laminaritriose to laminariheptaose.

Lutean (luteose) and oligosaccharides from lutean. Luteic acid, the malonic acid ester of lutean, was prepared according to the method of Anderson & Raistrick (1936) from *Penicillium luteum* Zuckal (L.S.H.T.M. no. P42) obtained from Mr G. Smith of the London School of Hygiene and Tropical Medicine. The lutean was isolated from the luteic acid according to the method of Stacey (Anderson, Haworth, Raistrick & Stacey, 1939). A series of β-1:6-linked oligo-

glucosaccharides was prepared by hydrolysing 0·5 g. of lutean in 0·5 N-H_2SO_4 at 95° for 4·5 hr. Under these conditions a trial experiment showed that the maximum amount of trisaccharide would be formed in this time. Chromatography as for the laminarin hydrolysate gave a similar result except that, as these oligosaccharides move more slowly, the largest observed after 2 days' irrigation was luteotetraose (gentiotetraose).

Enzyme preparations. (*a*) *Myrothecium verrucaria* (Alb. & Schwz.) Ditm. ex Fr., I.M.I. 45541 = QM 460 = U.S.D.A. 1334.2, from the Commonwealth Mycological Institute, Kew, Surrey, was grown on slopes of potato–carrot agar plus filter-paper strips for 9 days at 30°. The spore suspension obtained from these slopes was used to inoculate 25 ml. of culture medium (Siu, 1951) dispensed in 125 ml. flasks with cotton linters (1%) as the only carbon source. The flasks were incubated on a reciprocating shaker (120 strokes/min.) for 5–6 days. At the end of this time the mycelial-cellulose mat was removed and the culture filtrate centrifuged and dialysed in nitrocellulose sacs. A white flocculent precipitate appeared which was spun off and the clear fluid was freeze-dried to a white powder.

(*b*) The crop juice of *Helix pomatia* was collected and freeze-dried.

(*c*) Emulsin was prepared from sweet almonds according to Edman & Jorpes (1941) to the stage of the first ammonium sulphate precipitate.

(*d*) Fresh gentian roots (*Gentiana lutea* L.) were obtained from France through the courtesy of Wheeler and Huisking Ltd.. London. Root sections (200 g.) were mixed in a Waring Blendor with 300 ml. of water, squeezed through cotton cloth and the insoluble material was removed by centrifuging. The supernatant (260 ml.) was dialysed for 48 hr. on a rocking dialyser against tap water and then 24 hr. against distilled water in the cold room. A slight precipitate was spun off and the supernatant freeze-dried and stored in a desiccator.

(*e*) A commercial preparation of glucose oxidase ('DeeO'; Takamine Laboratory Inc., Clifton, New Jersey, U.S.A.) (7·5 g.) was dissolved in 125 ml. of water, dialysed, centrifuged at 20000 *g* and freeze-dried.

(*f*) Commercial mould enzyme preparations from *Aspergillus niger* 'E19' (Rohm and Haas Co., Philadelphia 5, Penn., U.S.A.); Carterzyme (H. W. Carter and Co., Coleford, Glos.); Saccharozyme and Clarase (Takamine Laboratory Inc., Clifton, N.J., U.S.A.); *Aspergillus aureus*, *A. flavus* and *A. niger* (Norman Evans and Rais Ltd., Manchester); and Pectinol 100D (Rohm and Haas Co., Philadelphia 5, U.S.A.) were obtained as stable powders. Before use they were dissolved in water to give 10% (w/v) solutions and allowed to stand several hours in a refrigerator with occasional stirring. The insoluble material was spun off and the supernatant dialysed until free from reducing sugars. The solutions were then freeze-dried and the powders stored in a desiccator under vacuum. Many of these enzyme preparations also contain α-glucosidase activity.

Methods

Paper partition chromatography. Qualitative and quantitative chromatograms were run on Whatman no. 1 paper by downward development in tanks similar to those described by Dent (1948). Two solvent systems were used: *n*-butanol–pyridine–water (6:4:3, by vol.) (Chargaff, Levine & Green, 1948) and *n*-propanol–ethyl acetate–water (6:1:3, by vol.) (Albon & Gross, 1952). The latter solvent mixture had a high resolving power for the higher oligosaccharides. Sugars were detected on the chromatograms by the benzidine–trichloroacetic acid reagent of Bacon & Edelman (1951). Silver nitrate was used according to Trevelyan, Procter & Harrison (1950). The lead tetra-acetate method of Buchanan, Dekker & Long (1950) was used to detect non-reducing derivatives of sugars. These are also detected by the $AgNO_3$ spray.

Quantitative chromatography was carried out essentially according to Bacon & Edelman (1951). The eluted sugars were estimated as glucose by the anthrone method (Trevelyan & Harrison, 1952) in solutions diluted to contain not more than 100 μg. of glucose/ml.

Paper electrophoresis of oligosaccharides. The apparatus used was essentially that of Consden & Stanier (1952). The paper was immersed in chlorobenzene to prevent evaporation and improve cooling. Electrophoresis was carried out on Whatman no. 1 paper in pH 10 borate buffer (7·44 g. of boric acid in 1 l. of 0·1 N-NaOH). A current of 1·5 mA/cm. width of paper was applied during 2·5 hr. The findings of Foster (1953) and of Aso & Hamada (1955) were used as an aid in the identification of compounds found.

For preparative work Whatman no. 3 MM paper was used. After spraying a guide strip the paper containing the sugars was cut out and eluted with water. The extracts were passed through a short column of Zeo-Karb 215 (The Permutit Co. Ltd., London) to remove the cations, and the boric acid was then removed by evaporating to dryness several times with 200 ml. portions of absolute methanol (Zill, Khym & Cheniae, 1953). Finally, the dry residue was dissolved in water and freeze-dried.

Chromatography on charcoal or charcoal–Celite mixtures. Mixtures of oligosaccharides were separated on charcoal or charcoal–Celite (Whistler & Durso, 1950) columns. The sugars adsorbed on the columns were eluted by a continuous gradient of aqueous ethanol produced by the method of Alm, Williams & Tiselius (1952), which gives an exponential rate of increase of ethanol concentration, or by the method of Parr (1954), which gives a linear rate of increase. Activated charcoal (B.D.H. Ltd.) or Speakman no. 130 (Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.) and Celite no. 513 (Johns-Manville and Co. Ltd., London) were used in preparing the columns. The preparation of the columns was greatly simplified by grading the charcoal and omitting the Celite. Sieving to give a cut between 50 and 200 B.S. mesh yielded a column which had approximately twice the capacity of the charcoal–Celite mixture was simpler to prepare, and ran more rapidly. Fractions were collected automatically (Crook & Datta, 1951) and analysed for total carbohydrate by the anthrone method. Fractions were concentrated under reduced pressure and finally freeze-dried and the dry powders stored in a desiccator.

Incubation procedure. The enzyme–substrate–buffer solutions were incubated in stoppered tubes in a water bath at 40°. Toluene was added if the experiment was to continue for longer than 8 hr. Samples to be analysed were removed by micropipette and applied in 30–50 μl. amounts, 5 μl. at a time, to the starting line of a paper chromatogram. To inactivate the enzyme 5 μl. portions of 0·01 M-$HgCl_2$ were previously applied at the starting points (Bealing & Bacon, 1953).

When the products were to be analysed quantitatively, 0·5 ml. samples were removed at suitable intervals from the

reaction mixture, 0·05 ml. of 0·04 M-$HgCl_2$ was added and the solution frozen in acetone–solid CO_2. The frozen solutions were stored at $-20°$ in stoppered tubes and at a convenient time were analysed by quantitative chromatography.

RESULTS

Except where indicated, the *Aspergillus niger* preparation, 'E 19', was used as the β-glucosidase source in the following experiments.

Action on cellobiose

To 0·8 ml. of 0·01 M acetate buffer, pH 5·0, containing 50 mg. of cellobiose was added 0·2 ml. of enzyme solution (10 mg. of freeze-dried preparation/ml.) and the mixture incubated at 40°. Samples (30 μl.) were removed at intervals and spotted on a chromatogram which was run as described, usually in butanol–pyridine–water. The chromatogram, when developed with benzidine–trichloroacetic acid reagent, showed at least six reducing compounds with R_G values greater or smaller than that of cellobiose (Fig. 1). The fastest-moving compound corresponded in position to the glucose marker.

Representative R_G values in butanol–pyridine–water of the most prominent spots, together with the designations adopted in this paper, are indicated in Fig. 2.

Chromatograms sprayed with the more sensitive $AgNO_3$ reagent (Fig. 5*a*) detected at least two other compounds with R_G values lower than the δ-compound, a third compound (β_1) with an R_G slightly greater than β and a fourth (ω_1) with R_G slightly greater than cellobiose. The β_1-compound was more evident when higher concentrations of cellobiose were used (see below).

Most of the enzyme solutions themselves showed only a faint reducing spot with benzidine–trichloroacetic acid reagent and this spot did not move from the starting line. A few, however, showed traces of a compound with the same mobility as glucose.

It can be seen from the chromatogram in Fig. 1 that the cellobiose gradually disappears, whereas the glucose spot increases in intensity. The concentrations of the low-R_G compounds and the ω-compound, on the other hand, reach a maximum and then decrease, so that after 3 hr. glucose and the α-compound were the only ones remaining in

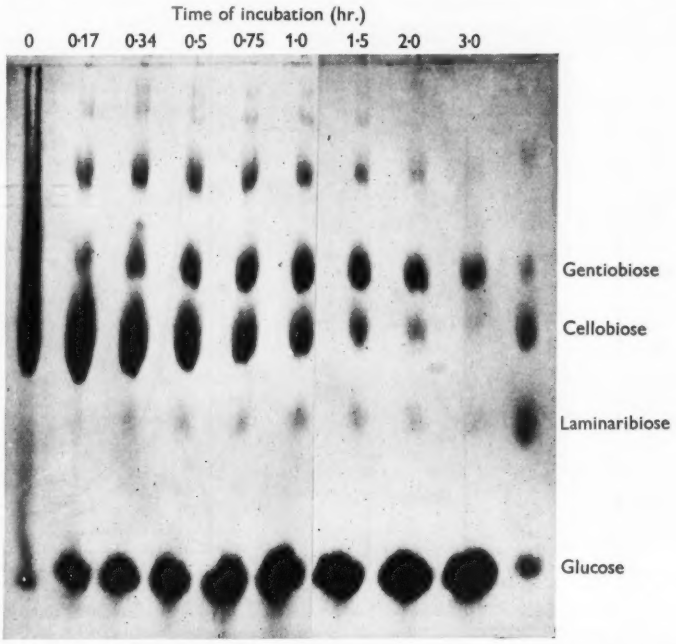

Fig. 1. Paper chromatogram of successive samples of the reaction mixture during the incubation of *A. niger* preparation ('E 19') with 5% (w/v) cellobiose in 0·01 M acetate buffer (pH 5·0) at 40°. Chromatogram developed with *n*-butanol–pyridine–water, sugars detected with benzidine–trichloroacetic acid reagent.

appreciable amounts. With 7·5 % (w/v) cellobiose as substrate and under the same conditions as before the reaction was followed quantitatively and the curves in Fig. 3 illustrate its course. The γ- and the δ-compounds were estimated together because of the small concentration of the latter. Glucose appeared rapidly, and almost simultaneously the β-compound, reaching a maximum early, to be followed by the $(\gamma+\delta)$- and later by the α- and ω-compounds. The β- and $(\gamma+\delta)$-compounds decreased most rapidly; the α- and ω-compounds were slower. Addition of 5 % glucose, as well as 7·5 % cellobiose, retarded the disappearance of the cellobiose and depressed the formation of the β-compound while increasing the α- and ω-compounds (Fig. 4). These observations suggest that α- and ω-compounds were formed by transfer to glucose, whereas the β-compound was formed by transfer to an oligosaccharide present in greatest concentration at the earliest stages of the reaction, namely cellobiose. The β-compound would then be a trisaccharide and the α- and ω-compounds disaccharides, a suggestion compatible with the mobilities of the α- and ω-compounds, which are identical with gentiobiose and laminaribiose markers.

Action on glucose

Oligosaccharides were also formed synthetically. Thus Fig. 5*c* shows the results of incubating 7·5 % (w/v) glucose under the same conditions as above with the enzyme for 28 hr. and spraying the chromatogram of the reaction mixture with $AgNO_3$. Cellobiose and the α- and ω-compounds (and ω_1-?) were present, but no component with an R_G lower than the α-compound; thus this synthesis does not account for all the oligosaccharides formed from cellobiose. A comparison of the time course of the formation of the α-compound from 7·5 % cellobiose, from 7·5 % cellobiose plus 5 % glucose and from 12·5 % glucose (Fig. 6) showed that the synthesis of the α-compound via glucose could not account for its rapid formation when cellobiose or a mixture of cellobiose and glucose was the substrate. Equilibrium was reached after 25–30 hr., and the final concentration of α-compound depended only on the total concentration of free and combined glucose.

Effect of glucose oxidase

Cellobiose (5 %, w/v) was treated under the conditions described above with the addition of 10 mg. of glucose oxidase and the reaction followed by paper chromatography. The glucose was converted almost entirely into gluconic acid, which was not detected by the benzidine–trichloroacetic acid reagent.

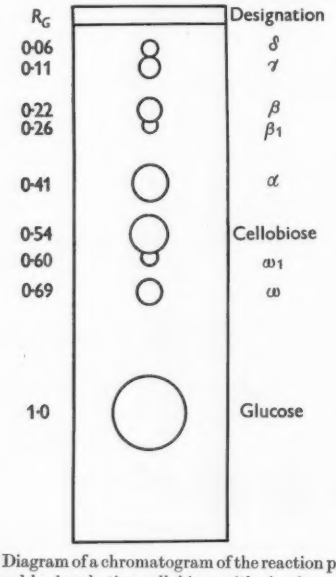

Fig. 2. Diagram of a chromatogram of the reaction products obtained by incubating cellobiose with *A. niger* preparation ('E 19'), showing their R_G values in *n*-butanol–pyridine–water and the designations used in the text.

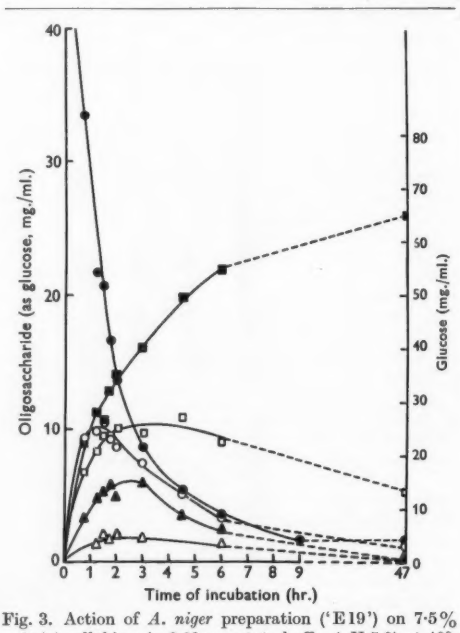

Fig. 3. Action of *A. niger* preparation ('E 19') on 7·5 % (w/v) cellobiose in 0·01 M acetate buffer (pH 5·0) at 40°. Oligosaccharides estimated as glucose. Concentrations in mg./ml. of incubation mixture. ●, Cellobiose; ■, glucose; □, α-compound; ○, β-compound; ▲, $(\gamma+\delta)$-compounds; △, ω-compound.

Spraying with lead tetra-acetate showed that the gluconic acid has an R_g similar to that of the γ-compound. To prevent its interference with the upper spots, therefore, it was removed by shaking the solution with the ion-exchange resin Biodeminrolit (The Permutit Co. Ltd., London). It was difficult to prevent the accumulation of glucose during the first 2 hr. of reaction, but it was reduced to a small fraction of the usual amount. At the same time, the α- and ω-compounds were almost absent, and so also was the γ-compound. Cellobiose remained in high concentration and the β- and δ-compounds appeared early in the reaction as usual. These results confirm that the α- and ω-compounds are formed by transfer to glucose, and suggest that, as the γ-compound disappears when the α-compound does, it is normally formed by transfer to the α-compound, whereas the δ-compound may be formed by transfer to cellobiose or, more probably, to the β-compound.

Effect of cellobiose concentration

These conclusions were further strengthened by the results of an experiment in which 0·2 ml. of enzyme (10 mg./ml.) was incubated at 40° with

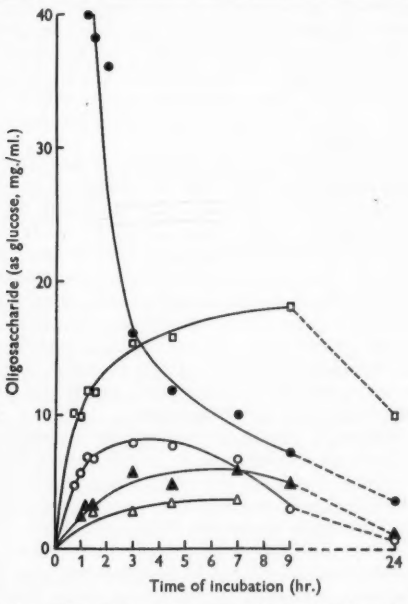

Fig. 4. Action of *A. niger* preparation ('E 19') on 7·5% (w/v) cellobiose plus 5·0% (w/v) glucose in 0·01 M acetate buffer (pH 5·0) at 40°. Oligosaccharides estimated as glucose. Concentrations in mg./ml. of the incubation mixture. Glucose not estimated. ●, Cellobiose; □, α-compound; ○, β-compound; ▲, (γ+δ)-compounds; △ ω-compound.

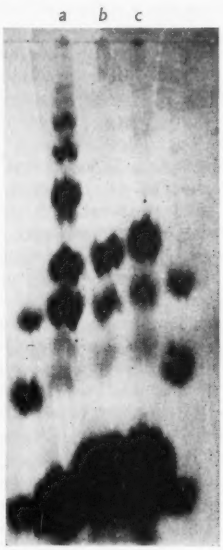

Fig. 5. Chromatogram comparing the composition of reaction mixtures obtained when *A. niger* preparation ('E 19') is incubated with (*a*) cellobiose, 5% (w/v), 1 hr.; (*b*) cellobiose, 5% (w/v), 24 hr.; (*c*) glucose 7·5% (w/v), 28 hr. in 0·01 M acetate buffer (pH 5·0) at 40°. Chromatograms developed in *n*-propanol–ethyl acetate–water for 4 days at 25°; sugars detected with $AgNO_3$ spray.

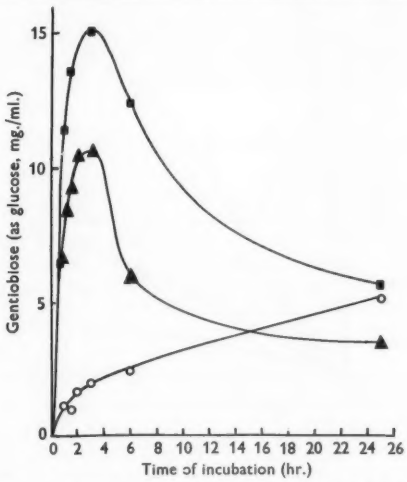

Fig. 6. Time course of gentiobiose formation during the incubation of *A. niger* enzyme ('E 19') with 12·5% (w/v) glucose (○), 7·5% (w/v) glucose plus 5% (w/v) cellobiose (■) and 5% cellobiose (▲) in 0·01 M acetate buffer (pH 5·0) at 40°. Concentrations in mg./ml. of incubation mixture.

0·8 ml. of 0·01 M acetate buffer (pH 5·0) containing cellobiose to give final concentrations ranging between 1 and 30 % (w/v). After 60 min. 0·05 ml. of 0·04 M-$HgCl_2$ was added and the mixture frozen in acetone–solid CO_2. The composition of the reaction mixture, after appropriate dilution of the higher concentrations, was determined by quantitative paper chromatography. It was noted that at the highest concentration of cellobiose an easily detected extra spot appeared with an R_G slightly greater than that of the β-compound. It has been referred to as β_1 and has a mobility corresponding to cellotriose. In this experiment it was estimated with the β-compound. Fig. 7 shows that glucose was liberated at very low concentrations of cellobiose whereas oligosaccharides were absent below 2·5 %. The α-compound made its appearance at low cellobiose concentrations and when there was an appreciable concentration of glucose. The amount of α-compound formed increased rapidly between 1 and 10 % cellobiose concentration with only a small increase thereafter; this course corresponded with that of glucose liberation, i.e. it behaved as though its concentration depended on the concentration of glucose rather than on that of cellobiose. On the other hand, the β-compound was not formed below 2·5 % cellobiose but continued to increase after the glucose and α-compound had ceased to accumulate rapidly. Again, these observations would be expected if the α-compound were formed by transfer to glucose and the β-compound by transfer to cellobiose.

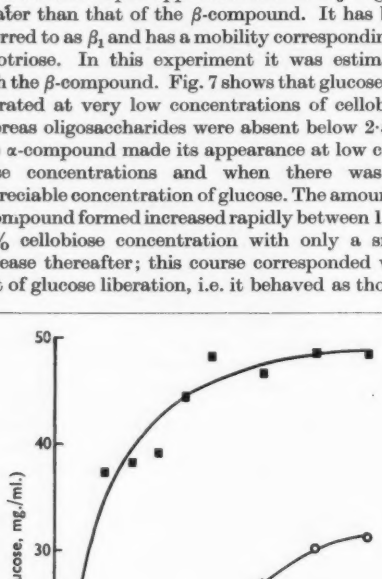

Fig. 7. Effect of concentration of cellobiose on the liberation of glucose and the formation of oligosaccharides. *A. niger* ('E 19') preparation incubated for 1 hr. with cellobiose solutions in 0·01 M acetate buffer (pH 5·0) at 40°. Oligosaccharides estimated as glucose. Concentrations in mg./ml. of the incubation mixture. ■, Glucose; □, α-compound; ○, β-compound; ▲, $(\gamma+\delta)$-compounds; △, ω-compound.

Separation and isolation of oligosaccharides

To 15 g. of cellobiose in 80 ml. of 0·01 M acetate buffer (pH 5·0) 20 ml. of enzyme solution (20 mg./ml.) was added, and the mixture incubated at 40°. After 2 hr. the reaction was stopped by the addition of 1 ml. of 0·04 M-$HgCl_2$ and heating at 100° for 3 min. The inactivated mixture was applied to a column of charcoal–Celite (250 g.:250 g.; 54·5 cm. × 5 cm.). Water (5 l.) was run through to remove the glucose formed. A linear gradient of ethanol (0–20 % ethanol in 20 l.) was then applied to the column. Fractions (50 ml.) were collected and the course of the elution was followed by anthrone estimation of the sugars in the fractions. The fractions were pooled on the basis of the elution curve, evaporated to a small volume and freeze-dried. Three fractions were obtained in this way: fraction I (0–5 % ethanol approx.), fraction II (5–10 % ethanol approx.), fraction III (10–20 % ethanol approx.). The three fractions were analysed by paper chromatography and electrophoresis in borate buffer.

Fraction I. Paper chromatography showed two components: one with the R_G (0·69) of the ω-compound and laminaribiose, the other with an R_G (0·60) slightly higher than cellobiose (0·54) and identical with that of the ω_1-compound and with sophorose. Electrophoresis showed two components: one migrating to the same position as laminaribiose, the second moving more slowly and to a position intermediate between laminaribiose and cellobiose, as does sophorose (cf. Aso & Hamada, 1955). As the amount of this fraction was small, no attempt was made to separate the components.

Fraction II. Paper chromatography showed two components: one with the R_G of cellobiose and the other with the R_G of the α-compound and gentiobiose. Electrophoresis also showed two components with mobilities consistent with gentiobiose and cellobiose. The high mobility of the borate complex of gentiobiose enabled it to be separated from the cellobiose complex, which moves only slowly. Fraction II (500 mg.) was fractionated by preparative electrophoresis as described. The fast-moving component (II *a*) was isolated and corresponded to gentiobiose and the α-compound on electrophoresis and chromatography, and showed $[\alpha]_D^{20} + 8°$ in water (*c*, 4) (gentiobiose has $[\alpha]_D^{20} + 9·6°$).

The acetate (acetic anhydride–pyridine) melted at 195° (corr.) and had $[\alpha]_D^{20} - 5°$ in $CHCl_3$ (*c*, 3). Gentiobiose β-octa-acetate has m.p. 196° (corr.) and $[\alpha]_D^{20} = -5{\cdot}4°$ in $CHCl_3$. The second slow-moving component (II*b*) corresponded to cellobiose in chromatography and electrophoresis.

Fraction III. Paper chromatography showed two main components with the same R_G values as the β- and γ-compounds. Electrophoresis showed one immobile and one fast-moving component. Fraction III (500 mg.) was separated into two components on the basis of their electrophoretic mobilities. The immobile component III*a* had the same mobility as the β-compound on chromatography but did not correspond in R_G to any member of the laminarin or lutean series. On complete acid hydrolysis (1·5 N-H_2SO_4, 5 hr. at 100°) it yielded only glucose (chromatographically); partial acid hydrolysis (0·33 N-H_2SO_4, 1 hr. at 80°) at concentrations which preclude transfer showed components corresponding chromatographically and electrophoretically to cellobiose, gentiobiose and glucose. Component III*a* showed $[\alpha]_D^{20} + 9{\cdot}2°$ in water (*c*, 6·8). It formed an acetate with m.p. approx. 205° and $[\alpha]_D^{20}$ approx. −13° in $CHCl_3$. The fast-moving component, III*b*, had the same R_G as the γ-compound and corresponded electrophoretically and chromatographically to the second member of the series of oligosaccharides obtained by the acid hydrolysis of lutean, namely gentiotriose. It was completely hydrolysed by acid to glucose (1·5 N-H_2SO_4, 5 hr. at 100°) and on partial acid hydrolysis (0·33 N-H_2SO_4, 1 hr. at 80°) gentiobiose and glucose were identified as the only products by chromatography and electrophoresis.

Action on other β-glucosides

Laminaribiose. Laminaribiose (5 %, w/v) was incubated with the enzyme under the same conditions as for cellobiose and the reaction followed chromatographically. The laminaribiose disappeared with the concomitant formation of glucose. Oligosaccharides appeared and disappeared during the reaction, as with cellobiose. The most rapidly formed was a compound with an R_G somewhat greater than the β-compound and intermediate between laminaritriose and laminaritetraose. By analogy with the β-compound it would be a β-1:6–1:3 trisaccharide and its R_G was compatible with this. Its appearance was closely followed by a compound, with the R_G of gentiobiose, which was formed in relatively large amounts. Both of these compounds practically disappeared after 24 hr. Traces of a compound with the R_G of cellobiose could be detected. Also detectable were compounds with lower R_G values.

Gentiobiose. Gentiobiose (5 %, w/v) was incubated under the same conditions as for cellobiose and the reaction followed chromatographically. The disappearance of gentiobiose was accompanied by the appearance of glucose. A trisaccharide with an R_G corresponding to that of the γ-compound and of gentiotriose had a transient appearance.

Methyl β-glucoside and salicin (saligenin β-glucoside). Methyl β-glucoside (5 %, w/v) and salicin (5 %, w/v) were incubated with the enzyme under the same conditions as for cellobiose and the course of the reaction was followed chromatographically. The benzidine–trichloroacetic acid reagent detected spots corresponding in mobility to glucose and gentiobiose. The silver nitrate reagent, which also detects glycosides, showed additional spots above gentiobiose. These were not methyl β-glucoside or salicin, since these compounds have mobilities greater than glucose.

Effect of the addition of galactose and xylose

Cellobiose (7·5 %, w/v) was incubated separately with D-galactose (5 %, w/v) and D-xylose (5 %, w/v), under the same conditions as for cellobiose; the reactions were followed by paper chromatography. In the presence of galactose an extra compound running between gentiobiose and β-compound was detectable. This persisted together with the α-compound when no cellobiose could be detected. The R_G of this compound is compatible with the structure 6-glucosylgalactose, which would be formed in a manner analogous to the α-compound. The amount of β-compound was reduced, as compared with that formed when cellobiose was incubated alone with the enzyme. In the presence of xylose a small amount of a compound running between laminaribiose and cellobiose appeared, but there was no other alteration in the usual pattern of spots.

Action of other enzyme preparations on cellobiose

Preparations from various microfungi, from the snail *Helix pomatia*, from sweet almonds and from fresh gentian roots (see Materials) were incubated with 5 % (w/v) cellobiose in 0·01 M acetate buffer (pH 5·0) at 40°. Toluene was added as antiseptic. Samples were removed at intervals up to 7 days, and analysed by paper chromatography. All these preparations were able to hydrolyse cellobiose and produce a series of compounds identical, as far as can be judged by chromatographic mobilities, with those produced by the *A. niger* preparation when incubated with cellobiose. The *Myrothecium verrucaria* preparation was unique in giving rise to very small amounts of gentiobiose and γ-compound, thus adding to the evidence that these two are closely related. The *Helix pomatia* preparation had the same order of activity as the *A. niger* ('E 19'), whereas of the other mould preparations *A. aureus* gave the next highest activity. The β-glucosidase activities

of most of the mould preparations were low. Emulsin had a high activity and upper spots could be detected only when appropriately diluted enzyme was used.

DISCUSSION

Identity of oligosaccharides

The ω-compound may be tentatively identified from electrophoretic and chromatographic data as laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucose). The other component (ω_1) of fraction I had an electrophoretic and chromatographic mobility compatible with sophorose (2-*O*-β-D-glucopyranosyl-D-glucose). The α-compound agrees in properties with gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose).

The β-compound behaves chromatographically as a trisaccharide and its kinetics of formation are compatible with this formulation. It has an electrophoretic mobility compatible only with the reducing glucose moiety being linked through the 4-position (cf. mobility of cellobiose). The products of partial hydrolysis indicate that the other glycosidic linkage must be 1:6. The low specific rotation suggests that the glycosidic linkages are of the β-configuration, as does the rotation of the acetate, which is also too negative for the known compound with β-1:4- and β-1:6-linkages, namely 4^2-β-glucosylgentiobiose* [*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 6)-D-glucose] whose $[\alpha]_D^{20}$ is $-10{\cdot}3°$ (Helferich & Schäfer, 1926). The β-compound would then have the structure 6^2-β-glucosylcellobiose (*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose). After the action of *A. niger* (strain 152) on cellobiose Barker *et al.* (1955) have isolated a trisaccharide with $[\alpha]_D^{20} + 10{\cdot}2°$ and with similar properties to the β-compound and have proposed the same structure. They also provide evidence that their compound must be 6^2- and not 6^1-β-glucosylcellobiose. The evidence cited here allows no such unambiguous formulation of the β-compound. However, on the basis of the general finding that most transfers are to non-reducing terminal residues and by analogy with the findings of Barker *et al.* the 6^2-β-glucosylcellobiose has been preferred.

The γ-compound is also a trisaccharide with properties compatible with the following structure: *O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 6)-D-glucose, i.e. gentiotriose. This compound has previously been reported by Lindberg & Macpherson (1954). Helferich & Schäfer (1926) and Helferich & Gootz (1931) have synthesized the hendeca-acetate of gentiotriose. The δ-compound (see above) has not been isolated, but chromatographically it behaves as a tetrasaccharide (not gentiotetraose), and its electrophoretic behaviour suggests that the reducing glucose moiety is linked through the 4-position. The order of appearance of the lower R_G compounds under certain conditions, e.g. at pH 6·8 and 7·5, suggests that the β- and δ-compounds are related. The experiments with glucose oxidase also confirm that the δ-compound depends on the β- and not on the α- or γ-compounds, i.e. that it is probably formed by transfer from cellobiose to the β-compound. The most probable structure for the δ-compound on the basis of the mechanism put forward below is 6^2-β-gentiobiosylcellobiose (*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose).

* The shortened names are coined by assuming the sugar to be a derivative of the largest oligosaccharide within the molecule which contains the same type of linkage and the free reducing-end group. The superscript numeral refers to the glucose unit undergoing substitution, the reducing-end member being numbered 1, etc.

General

Enzymes from various sources catalyse the transient formation of glucose oligosaccharides during the hydrolysis of cellobiose and other β-glucosides. The oligosaccharides appear to be all β-linked, i.e. the steric configuration is retained. During the hydrolysis of methyl β-glucoside by emulsin (Bunton, Lewis, Llewellyn, Tristram & Vernon, 1954) and of salicin by an unspecified β-glucosidase (Springhorn & Koshland, 1955) cleavage of the glucosidic link occurs between the glucose carbon and the oxygen. Transfer from these compounds have been demonstrated with most of the enzymes used in the present work, and it is therefore likely that the same mechanism can be extended to cover them, i.e. that they are transglycosylations (cf. Hehre, 1951).

Transfer could proceed to any carbon atom on the acceptor with a free hydroxyl group, and if the acceptor happens to be free glucose any one of the six possible glucopyranose disaccharides with a β-linkage could be formed. Transfer to higher saccharides is of course restricted by the elimination of those hydroxyls already occupied by glucosidic links. It has also been generally observed that transfers are restricted to the non-reducing end of a chain, although the recent work of Bailey, Barker, Bourne & Stacey (1955) shows that other possibilities must be kept in mind.

In the present work direct evidence was obtained for transfer to all positions of glucose acceptor except C-1. The proportions of the various isomers formed at equilibrium (47 hr. values, Fig. 3) appear to fall in the same order as observed by Peat *et al.* (1952) for the five isomeric β-linked glucose disaccharides based on the yield in the condensation reaction (1:6 > 1:4 > 1:3 > 1:2 > 1:1). An identical

series is obtained on the basis of yields of acid-reversion products from glucose (Thompson, Anno, Wolfrom & Inatome, 1954; Wolfrom & Thompson, 1955). In the present work the position of sophorose in the series depends on the observation that sophorose is no longer detectable at equilibrium and hence must follow laminaribiose. The present data suggest that the relative rates of formation of the linkages is approximately in the same order, 1:6-linkages being formed faster than any others, whereas the rate of formation of 1:3-links is low. It is difficult to obtain good evidence for the relative speed of formation of 1:4-links. A tentative conclusion may be drawn from a comparison of Figs. 3 and 4. In the initial stages of the reaction, addition of glucose has retarded the disappearance of cellobiose without appreciably increasing the rate of appearance of laminaribiose. This suggests that the retardation is due to the transfer more rapidly to the 4-position of glucose than to the 3-position. The same predominance in transfer to the primary hydroxyl at C-6 on a pyranose residue occurs in other enzyme transfers, e.g. amylose → amylopectin, dextrin → dextran, sucrose → dextran, maltose → panose (6^2-α-glucosylmaltose), and lactose → 6^{Gal}-α-galactosyl-lactose, and also in the transfer of fructose residues by yeast invertase to the primary hydroxyl group of glucose or to one of the three primary hydroxyl groups of sucrose. However, in many instances it was not clear whether transfer was to primary hydroxyl groups exclusively or whether the analytical methods were not sensitive enough to detect other products. Thus recently Wilham, Alexander & Jeanes (1955) have shown, using periodate oxidation techniques, that enzymically synthesized dextrans also contain varying proportions of 1:4- and 1:3-like linkages, and Wolfrom & Thompson (1955) have detected α-1:3-links (nigerose) in hydrolysates of amylopectin. 1:3-Links have also been detected in the reaction products of enzymes on maltose (Barker & Carrington, 1953; Matsuda & Aso, 1953; see also the chromatograms of Pazur & French, 1952, which show a compound in the correct position for a 1:3-linked disaccharide although these authors do not comment on it).

On the basis of these conclusions, and taking into account the structures which have been deduced for the various compounds observed on the chromatograms, the following tentative scheme may be drawn up to account for the reactions occurring when cellobiose is broken down.

Initially cellobiose is the only glucose donor available, but both cellobiose and water are available acceptors:

$$G\beta\text{—}4G + H_2O \rightarrow 2G, \quad (1)$$

$$G\beta\text{—}4G + G\beta\text{—}4G \rightarrow G\beta\text{—}6G\beta\text{—}4G + G. \quad (2)$$

Reaction (2) results in the formation of the β-compound, and both give rise to free glucose. It would be expected that small quantities of cellotriose (Gβ—4Gβ—4G) would also be formed at this stage together with traces of Gβ—3Gβ—4G, but the great predominance of 1:6-transfers results in their concentration being too low to detect under the present conditions except with high concentrations of cellobiose (25–30 %). There then appears a small amount of a compound with a chromatographic mobility compatible with cellotriose.

The free glucose liberated in reactions (1) and (2) can also act as acceptor and, since it rapidly reaches high concentrations, transfers to all positions except C-1 are demonstrable, with 1:6-transfers again predominating. In this way the disaccharides gentiobiose, more cellobiose, laminaribiose and sophorose are formed by transfers to C-6, C-4, C-3, and C-2 respectively. All these products of transfer can themselves act as acceptors. Then from the β-compound

$$G\beta\text{—}4G + G\beta\text{—}6G\beta\text{—}4G \rightarrow G\beta\text{—}6G\beta\text{—}6G\beta\text{—}4G + G, \quad (3)$$

and from gentiobiose

$$G\beta\text{—}4G + G\beta\text{—}6G \rightarrow G\beta\text{—}6G\beta\text{—}6G + G. \quad (4)$$

The products of reactions (3) and (4) are respectively the δ- and γ-compounds. Again, trisaccharides resulting from transfer to laminaribiose and sophorose would be expected, but are below the limits of detection. That they can be formed, however, is shown by the experiments in which laminaribiose was the starting material. Here a Gβ—6Gβ—3G trisaccharide is formed. When glucose oxidase is added to the reaction mixture to remove the glucose as it is formed, new disaccharides and hence γ-compound are no longer formed. Transfers to sugars and sugar derivatives other than glucose are also possible. Methyl β-glucoside, salicin, D-galactose and D-xylose have been shown to be acceptors during the present experiments.

Thus complex series of oligosaccharides are built up and the chromatographic picture obtained depends on three factors: (i) the great preponderance of transfers to C-6; (ii) the linkage in the starting disaccharide; (iii) the initial concentration of the starting material. The first ensures that chains of 1:6-links predominate. The second determines the linkage at the reducing end of the chains. However, there is a complication, since the presence of free glucose together with factor (i) ensures that gentiobiose is always present and therefore that there are always chains which end in 1:6-linkages, whatever the linkage in the primary disaccharide. The third factor determines whether there are appreciable amounts of linkages other than 1:6 in parts of the chain distant from the terminal reducing-end linkage. Thus it is only with high concentrations

of starting material that the intermediates reach sufficient concentrations for the second link to be other than 1:6, and as 1:4 is the next most probable by a good margin this is the most likely configuration for that linkage. The probability that linkages other than 1:6 would occur further away than one from the terminal linkage is small, even with high initial concentrations (25–30 %) of disaccharide.

Alternative routes are also possible. Thus some gentiobiose undoubtedly arises from glucose in strong sugar solutions, but the following considerations show that free glucose plays a minor role in the other syntheses. The β-compound is not formed from glucose alone in the presence of the enzyme (Fig. 5c), and the addition of glucose in the presence of cellobiose actually diminishes formation of β-compound, simultaneously increasing that of gentiobiose (Fig. 4), i.e. glucose competes successfully for the transferred radicals. Moreover, when cellobiose is the substrate, glucose continually increases in concentration throughout the course of the reaction, whereas the oligosaccharides early reach maxima which are many times their final equilibrium concentrations (Figs. 3, 4 and 6). Further, as is shown in Fig. 6, the initial rate of formation of α-compound from cellobiose is 20–30 times as high as from an equivalent concentration of glucose.

Thus it seems probable that the first route pictured above is the most important for the formation of β-compound. It is, however, possible that higher saccharides are formed by transfer of more than one residue at a time, e.g. gentiotriose could be formed from β-compound by transfer of a gentiobiosyl residue to glucose (eqn. 5):

$$G\beta\text{—}6G\beta\text{—}4G + G \rightarrow G\beta\text{—}6G\beta\text{—}6G + G. \quad (5)$$

Gentiobiose might arise by a similar transfer to water. This would be analogous to the *D*-enzyme of potato, which has been shown to transfer two or more glucose residues of a maltodextrin substrate to a suitable acceptor (Peat, Whelan & Rees, 1956). No evidence for or against such transfers can be adduced from the present experiments. The upper limit of chain length for acceptors is not known, but oligosaccharides of at least six units have been detected in these experiments. In addition to a diminishing donor concentration the acceptor-chain length may be a factor in determining the point at which the hydrolytic aspect of the reaction becomes overwhelming.

In the preceding discussion it has been assumed that water was an acceptor competing with sugar for the transferred glucosyl radicals, and this assumption has been shown to be compatible with the observed reactions. However, no evidence is available to show whether or not this competition is a result of the action of two different enzymes or a series of enzymes, or whether hydrolysis and transfer are both catalysed by a single enzyme. Although the latter is the simpler picture and appears to be true for β-glucuronidase (Fishman & Green, 1956), the identity of hydrolytic and transfer enzymes in our unpurified preparations must rest on indirect evidence (cf. Doudoroff, Barker & Hassid, 1947; Bealing & Bacon, 1953; Takano & Miwa, 1950, 1953). Studies of the pH–activity relationships and the results of fractionation of the β-glucosidase of *A. niger* (Stone, 1954) suggest that two components with β-glucosidase activity are present and that each fraction possesses both hydrolytic and transfer activity, although the ratio of these activities is different. This may be compared with the finding of Durell & Fruton (1954) that although trypsin and papain both catalyse transamidation reactions with the same substrates the efficiency of the transfer is different. However, there is no doubt that transfer is kinetically favoured compared with hydrolysis, since in a 5 % cellobiose solution the molar concentration of water is 400 times that of the sugar.

Similar difficulties arise in trying to decide whether a given enzyme is specific for the transfer from one particular carbon atom to another, or whether a single enzyme can catalyse all of the transfers demonstrated in this work. The simplest assumption would be that it can, and this would not be incompatible with any of the suggested reaction mechanisms. However, caution is necessary, since the discovery of trehalase in *A. niger* (Bourquelot, 1893) and its differentiation from the maltase of the same organism, by their different heat stabilities, suggests at least that enzymes with varying preferences may exist even if the specificities are not absolute. Moreover, Bealing & Bacon (1953) and Edelman (1954) have evidence that yeast and mould invertases, both fructosidases, produce a qualitatively different mixture of oligosaccharides. Also Barker *et al.* (1955) find with their resting-cell preparations of *A. niger* (strain 152) that laminaribiose appears before gentiobiose, whereas the opposite is true in the present experiments.

In the light of the present observations, it is possibly not without significance that a number of naturally occurring oligosaccharides, e.g. raffinose, gentianose, stachyose and verbascose, have structures in which one or more monosaccharide residues are attached to the C-6 hydroxyl group of sucrose. It is possible that gentianose arises from sucrose by the activity of the β-transglucosylase which, as the present work shows, occurs in gentian roots. Raffinose, stachyose and verbascose form a homologous series of α-1:6-galactosides of sucrose and have been found to occur together in *Verbascum thapsiforme* Schrad. (Hérissey, Fleury, Wickström, Courtois & Le Dizet, 1954; see also French, 1954)

and may be synthesized by enzymes with similar specificities.

The ability of alcohols to act as acceptors in the presence of β-glucosidases (Rabaté, 1935; Takano & Miwa, 1950; Jermyn & Thomas, 1953) also suggests that many of the heterosidic glycosides occurring in plants may be formed by transfer, especially as they are often accompanied by enzymes able to hydrolyse them. Thus Miller (1943) was able to show that *o*-chlorophenol and ethylene chlorohydrin, both potential glucose acceptors, were able to induce the formation of *o*-chlorophenyl β-gentiobioside and 2-chloroethyl β-glucoside in gladiolus corms, although these do not normally contain gentiobiose.

The study of transfer reactions involving β-glucosides naturally raises the question whether β-linked polysaccharides are synthesized by this route. This would imply an enzyme with an affinity for oligosaccharide acceptors that is higher than those studied in this work. Further, the degree of homogeneity of the linkages will depend on the transfer specificity of the enzyme. Thus enzymes with the degree of transfer specificity demonstrated in the present work would account for the formation of polysaccharides such as lutean, which is reported to contain 1:6-, 1:3- and 1:4-linkages (Lloyd, Pon & Stacey, 1956). On the other hand, from what we know of the structure of cellulose, its synthesis, if it is by a transfer mechanism, would require an enzyme with an absolute transfer specificity.

SUMMARY

1. During the hydrolysis of cellobiose to glucose by an enzyme preparation from *Aspergillus niger*, there is a transient formation of a number of glucose oligosaccharides.

2. Three disaccharides and two trisaccharides have been isolated and identified. A third trisaccharide and a tetrasaccharide were also observed in chromatograms of the reaction mixture.

3. Only the disaccharides were detected when the *A. niger* preparation was incubated with glucose under the same conditions.

4. Oligosaccharides were also produced during the incubation of the *A. niger* preparation with laminaribiose, gentiobiose, methyl β-glucoside and salicin.

5. The effects of cellobiose concentration, and of the presence of glucose, galactose or xylose, and glucose oxidase on the formation and nature of the oligosaccharides were investigated.

6. Enzyme preparations from other microfungi and from *Helix pomatia*, sweet almonds and gentian roots showed a qualitatively similar formation of oligosaccharides during cellobiose hydrolysis.

7. The formation of oligosaccharides is interpreted in terms of an enzyme-catalysed transference of glucosyl residues from cellobiose and other β-glucosides to suitable acceptors. Hydrolysis results when water is the acceptor.

8. The factors influencing the nature and amounts of oligosaccharides formed and the general significance of transferase reactions are discussed.

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The Isolation of a Ketonic Dihydroxy Kober Chromogen from the Urine of Pregnant Women

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In 1943 Pincus & Pearlman briefly referred in a review article to evidence obtained by them 'for the presence of an oestrogen in human pregnancy urine which is ketonic and contains a hydroxyl group other than the usual phenolic one'. Experimental details of this work were not published, but subsequently Huffman & Grollman (1947) suggested that this ketonic oestrogen might be 16-oxo-oestradiol-17β (oestra-1:3:5-triene-3:17β-diol-16-one), which previously had been prepared from oestrone by Huffman (1942) (cf. Huffman & Lott, 1948); it was also suggested by these authors that 16-oxooestradiol-17β might be an intermediate in the metabolic conversion of oestrone (oestra-1:3:5-trien-3-ol-17-one) into oestriol (oestra-1:3:5-triene-3:16α:17β-triol). More recently Migeon (1953) has reported the detection in extracts of acid-hydrolysed human urine of a H_2SO_4–fluorogen which behaved like 16-oxooestradiol-17β on counter-current distribution in two different solvent

systems; but since 16-oxooestradiol-17β added to urine could not be subsequently recovered after subjection of the latter to the usual acid hydrolysis and extraction procedures which were employed, it was concluded that the unidentified H_2SO_4–fluorogen was probably not identical with the former.

The suggestion that 16-oxooestradiol-17β might be an intermediate in the metabolism of the oestrogens was recently readvanced by Marrian & Bauld (1955) when they isolated 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol) from the urine of pregnant women. Although, at that time, the possibility that the isolated substance was an artifact had not been excluded (cf. Watson & Marrian, 1956), it was nevertheless tentatively suggested that 16-oxooestradiol-17β might be the common metabolic precursor of oestriol and 16-*epi*oestriol.

Following up this suggestion Watson & Marrian (1955)* examined the urine of pregnant women for the presence of this hypothetical ketonic precursor of the two epimeric triols; they were able to detect a Kober chromogen in ether-soluble ketonic–phenolic fractions, which was more 'polar' than oestrone, and which was indistinguishable from 16-oxooestradiol-17β by chromatography in the system 70 % (v/v) methanol in water on Celite–25 % (v/v) ethylene dichloride in benzene, and by countercurrent distribution in the system 20 % (v/v) *n*-hexane in benzene–50 % (v/v) ethanol in water. In these experiments preliminary enzymic hydrolysis of the urine gave yields of the unknown Kober chromogen about 2–3 times higher than those obtained after conventional hot-acid hydrolysis; and since 16-oxooestradiol-17β was found to be labile to hot-acid treatment in urine this finding indicated a further similarity between the urinary Kober chromogen and this compound.

Although it was believed by Watson & Marrian (1955) that the unknown ketonic Kober chromogen (KC-5) was 16-oxooestradiol-17β, it was appreciated that the identity of the former could only be established with certainty after isolation. The work described in the present paper was accordingly undertaken.

RESULTS

Isolation of KC-5

Partition chromatographic analysis of ether-soluble ketonic–phenolic fractions obtained from batches of pooled late-pregnancy urine after hydrolysis with enzymic preparations from *Patella vulgata* showed these fractions to contain 3·4–4·4 % by weight of oestrone and 14–22 % by weight of KC-5 (calc. as oestriol) (Table 1). The most effective method of those tried for the further concentration of KC-5 in such fractions was found to be chromatography on a large Celite column with the system 70 % (v/v) methanol in water–20 % (v/v) *n*-hexane in benzene. By this method, for instance, a concentrate containing 82 % of KC-5 was obtained from batch III. The isolation of crystalline material from such concentrates was achieved by washing with a small volume of chilled methanol and then crystallizing from methanol at $-20°$. The yield of recrystallized material was poor, but further quantities of crystalline material were obtained from the methanolic washing and mother liquor by evaporation to dryness and re-treating with small volumes of chilled methanol.

The recrystallized KC-5 melted at 236·5–238·5°, and the melting point was not significantly depressed after admixture with 16-oxooestradiol-17β (m.p. 238–241°). It gave C and H analyses in excellent agreement with those required for a compound of the formula $C_{18}H_{22}O_3$. However, it was clear that KC-5 could not be identical with 16-oxooestradiol-17β since it had $[\alpha]_D^{15} + 143°$ in ethanol (cf. 16-oxooestradiol-17β, $[\alpha]_D^{12} - 89°$ in ethanol). Furthermore, in contrast to 16-oxooestradiol-17β, it developed its maximum colour in the Kober reaction with no heating in the first stage (Fig. 1),

Table 1. *Ketonic–phenolic fractions from enzyme-hydrolysed late-pregnancy urine: oestrone and KC-5 contents*

Batch	Vol. of urine (l.)	Wt. of ketonic–phenolic fraction (mg.)	Oestrone content of fraction (%)	KC-5 content (%)
I	75	207	3·5	15
II	101	377	3·5	14
III	100	284	3·4	22
IV	95	274	4·4	15

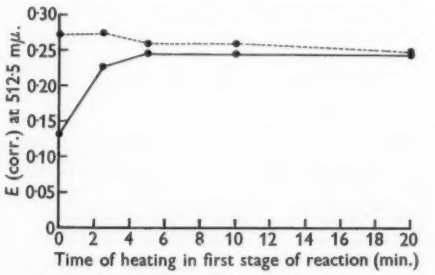

Fig. 1. Effect of variation in time of heating in the first stage of the Kober reaction on intensities of final colours developed by 16-oxooestradiol-17β (●—●) and KC-5 (●- - -●).

* The results obtained by Watson & Marrian (1955) were not reported in full detail. It is regretted that owing to limitations of space it is not possible to give these results in detail in the present paper.

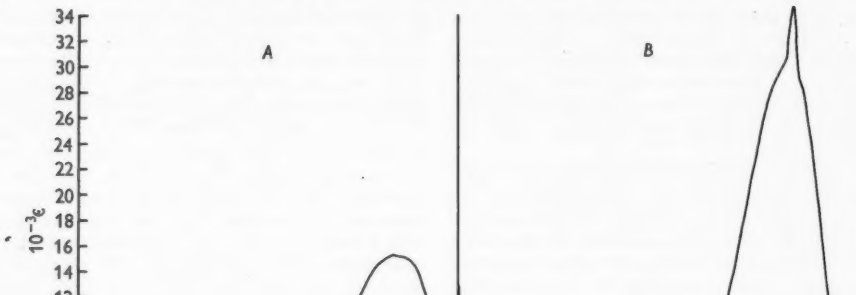

Fig. 2. Absorption spectra of H_2SO_4 solutions of 16-oxooestradiol-17β and KC-5 after 2 hr. at 24°. *A*, 16-Oxooestradiol-17β: $8{\cdot}39 \times 10^{-5}$M; *B*, KC-5: $8{\cdot}74 \times 10^{-5}$M.

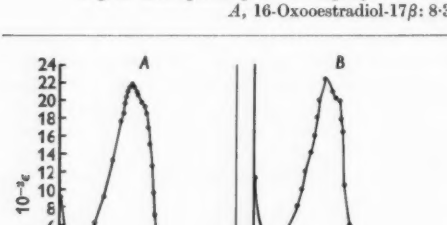

Fig. 3. Ultraviolet absorption spectra of 16-oxooestradiol-17β and KC-5 in ethanolic solution. *A*, 16-Oxooestradiol-17β: $1{\cdot}67 \times 10^{-4}$M; *B*, KC-5, $1{\cdot}75 \times 10^{-4}$M.

while the spectrum of its solution in conc. H_2SO_4 differed markedly from that of a similarly prepared solution of 16-oxooestradiol-17β (Fig. 2).

Characterization of KC-5

General. On acetylation KC-5 yielded a diacetate which melted at 166–167° (cf. 16-oxooestradiol-17β diacetate, m.p. 132·5–134°). This suggested the possibility that KC-5 might be 6-oxooestradiol-17β, the acetate of which melts at 173–175° (Longwell & Wintersteiner, 1940). However, this possibility was excluded by the ultraviolet-absorption spectrum in ethanol of KC-5, which was found to be virtually identical with those of 16-oxooestradiol-17β (Fig. 3), oestriol, 16-*epi*oestriol and oestrone, but markedly different from that of 6-oxooestradiol-17β as recorded by the above-mentioned authors.

In the 'blue tetrazolium' test carried out according to Mader & Buck (1952), KC-5 showed marked reducing power, suggesting that it was an α-ketol. However, the intensity of the colour developed in this test was only about one-third of that of the colour developed by an equivalent amount of 16-oxooestradiol-17β. This finding suggested the possibility that KC-5 might be a 16-hydroxyoestrone, since it had previously been found (Fotherby & Marrian, unpublished work) that 16α-hydroxyandrost-4-ene-3:17-dione (Fried, Thoma, Perlman, Herz & Borman, 1955) gives a considerably less intense colour in this test than those given by either 16-oxooestradiol-17β or pregn-4-ene-17α:21-diol-3:20-dione. The dextrorotation of KC-5 was consistent with the presence of a 17-oxo group (cf. Barton & Klyne, 1948), while the finding that the compound gave a negative Zimmermann reaction was consistent with the presence of 16-hydroxy-17-oxo grouping.

Sodium borohydride reduction of KC-5. To investigate the possibility of KC-5 being one or other of the two epimeric 16-hydroxyoestrones a sample of the isolated substance was reduced with sodium borohydride. Since by this method of reduction oestrone yields oestradiol-17β exclusively (Biel, 1951), it was expected that KC-5 would yield either oestriol or 16-*epi*oestriol, depending on whether the C-16 hydroxyl group of the former had the α- or β-configuration. Unfortunately, owing to the shortage

of available material this experiment had to be carried out on a sample of KC-5 which had been recovered from the mother liquors of the analytical sample.

On chromatographing a portion of the reduction product in the system 70% (v/v) methanol in water–ethylene dichloride the presence of two components in approximately equivalent amounts was unexpectedly revealed, and the elution characteristics of the less and more 'polar' of these were closely similar to those of 16-*epi*oestriol and oestriol respectively (Fig. 4). In view of this finding the main bulk of the reduction product was separated into its two components on a larger partition column with the same solvent system.

After recrystallization the less 'polar' component melted at 273–276°, and the melting point was not depressed after admixture with 16-*epi*oestriol (m.p. 284–285°). Because of the considerable difference in the melting points this was not accepted as completely satisfactory evidence of the identity of this reduction product with 16-*epi*oestriol. However, on acetylation the reduction product yielded an acetate, m.p. 151–152°, and the mixed melting point of this with 16-*epi*oestriol triacetate (m.p. 151–152°) showed no depression. Accordingly, there could be no doubt that this reduction product was identical with 16-*epi*oestriol.

The more 'polar' reduction product of KC-5 melted at 277–279° after recrystallization, and the melting point was not depressed after admixture with oestriol (m.p. 279–280°). It yielded an acetate, m.p. 125–128°, and the mixed melting point with oestriol triacetate (m.p. 126–128°) showed no depression. Accordingly, this second reduction product of KC-5 was clearly identical with oestriol.

On the reasonable assumption that no inversion of either a C-16 or a C-17 hydroxyl group occurs during sodium borohydride reduction, the only α-ketol which could give rise to oestriol when reduced by this method is 16α-hydroxyoestrone. Accordingly, it can be concluded that the latter was present in the sample of KC-5 used in the reduction experiment.

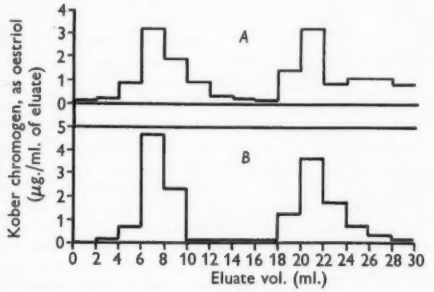

Fig. 4. Analytical column partition chromatograms (70% methanol in water–ethylene dichloride) on *A*, sodium borohydride reduction product from KC-5, and *B*, a mixture of 15·5 μg. of 16-*epi*oestriol and 16·3 μg. of oestriol.

On the other hand, either 16β-hydroxyoestrone or 16-oxooestradiol-17β could give rise to 16-*epi*oestriol by sodium borohydride reduction, and, indeed, it has been shown by Huffman & Lott (1955) that 16-*epi*oestriol is formed in nearly quantitative yield when 16-oxooestradiol-17β is reduced by this procedure. It seems probable, therefore, that the sample of KC-5 used in the reduction experiment was a mixture either of 16α-hydroxyoestrone and 16β-hydroxyoestrone or of 16α-hydroxyoestrone and 16-oxooestradiol-17β, although the alternative explanation, that a partial rearrangement of 16α-hydroxyoestrone to 16-oxooestradiol-17β occurred during the reduction, cannot be excluded. As the sample of KC-5 used for the reduction was material recovered from the mother liquors of the analytical sample, it is not unlikely that it was grossly impure. The diacetate of KC-5, which was prepared from a mixture of recrystallized material and material recovered from the mother liquors, had a melting point 13° lower than that reported for synthetic 16α-hydroxyoestrone diacetate by Leeds, Fukushima & Gallagher (1954). This finding is consistent with the view that the KC-5 recovered from the mother liquors contained in addition to 16α-hydroxyoestrone a considerable proportion either of 16β-hydroxyoestrone or of 16-oxooestradiol-17β.

While there is no reason to believe that the recrystallized KC-5 was a pure substance, it seems probable that the principal component in it must have been either 16α-hydroxyoestrone or 16β-hydroxyoestrone, and not the laevorotatory 16-oxooestradiol-17β. If the principal component in it was 16α-hydroxyoestrone, the accompanying impurity, which would presumably become concentrated in the mother liquor, could have been either 16β-hydroxyoestrone or 16-oxooestradiol-17β. On the other hand, if the principal component in it was 16β-hydroxyoestrone, the accompanying impurity must have been 16α-hydroxyoestrone.

Until a larger amount of more rigorously purified KC-5 has been examined a definite decision between these possibilities cannot be made. However, since Cooley, Ellis, Hartley & Petrov (1955) have shown that alkaline hydrolysis at room temperature of androst-5-ene-3β:16α-diol-17-one diacetate yields androst-5-ene-3β:17β-diol-16-one it is not improbable that if 16α-hydroxyoestrone had been present in the original urinary extracts the analogous rearrangement of 16α-hydroxyoestrone to 16-oxooestradiol-17β might have occurred to some extent during the extraction of the phenolic fraction with aqueous alkali. The view that the isolated KC-5 was

a mixture of 16α-hydroxyoestrone and artifactually produced 16-oxooestradiol-17β would seem to be entirely consistent with all the available evidence, and is therefore the favoured one.

EXPERIMENTAL

Methods

Enzymic hydrolysis of urine. Urine specimens were acidified to pH 4·7 with acetic acid, buffered to this pH by the addition of one-tenth of their volume of M acetate buffer, and incubated at 37° with the enzyme preparation for a period of 48 hr. In every instance half of the total amount of enzyme used was added initially and half after 24 hr.

Analytical partition chromatograms. The procedures based on those of Bauld (1955) and described by Marrian & Bauld (1955) and Watson & Marrian (1956) were employed.

Kober reaction. The method of Brown (1952), as modified by Bauld (1954), was employed, Bauld's 'oestriol reagent' being used. All determinations of Kober chromogens are expressed as oestriol equivalents.

Melting points. The melting points of 16-oxoestradiol-17β, KC-5, oestriol and 16-epioestriol were determined in sealed, evacuated capillary tubes, while for those of the acetates of these substances a microscope hot-stage was employed. The same thermometer was used for all determinations and the values given are uncorrected for emergent stem. Although corrected melting points were given for oestriol and 16-*epi*oestriol in a recent paper by two of the present authors (Watson & Marrian, 1956) we are now of the opinion that the method of correcting for emergent stem with the aid of pure standard compounds which was used is unreliable for temperatures much above 200°, because of the lack of reliable data for corrected melting points on suitable compounds.

Materials

16-*Oxooestradiol*-17β. This was prepared from oestrone by the method of Huffman (1942) and Huffman & Lott (1948), and was crystallized once from 50% (v/v) aqueous acetic acid and once from dilute methanol. The m.p. was 239–241° and it had $[\alpha]_D^{12} - 89°$ (0·600% in ethanol). It yielded a diacetate, m.p. 132·5–134°.

Oestriol. The preparation described by Marrian & Bauld (1955) was used. The m.p. was 279–280° and it had $[\alpha]_D^{19} + 61°$ (0·296% in ethanol). It yielded a triacetate, m.p. 126–128°.

16-epi*Oestriol.* The preparation described by Watson & Marrian (1956) was used. The m.p. was 284–285°, and it had $[\alpha]_D^{15} + 76°$ (0·297% in ethanol). It yielded a triacetate, m.p. 151–153°.

Enzyme preparations. These were crude acetone-dried powders obtained from *Patella vulgata* by the method of Dodgson & Spencer (1953). They were standardized for β-glucuronidase activity with phenolphthalein glucuronide as substrate by the method of Fishman (1948). The sulphatase activities of these preparations were not determined.

Miscellaneous. The solvents and Celite (Celite 535, Johns Manville and Co., Ltd., London) used were purified by the same methods employed by Watson & Marrian (1956).

Isolation of KC-5 from pregnancy urine

Late-pregnancy urine was enzymically hydrolysed in batches of 8–16 l. with amounts of the enzyme equivalent to about 1000000 Fishman units/l. of urine. The hydrolysed mixture was extracted once with an equal volume of ether after the addition of 600 g. of NaCl/4 l. of urine, the extract washed once with one-tenth of its volume of 5% (w/v) $NaHCO_3$ and then extracted twice with one-eighth volume of N-NaOH. The NaOH extract was partly neutralized by the addition of 5N-H_2SO_4, and then neutralization (to phenolphthalein) completed by passing in CO_2. The extraction with NaOH and the subsequent neutralization of the extract were carried out as rapidly as possible to minimize losses of KC-5 which occur in strongly alkaline solutions.

The neutralized extract was extracted twice with equal volumes of ether, the extract washed twice with one-quarter volumes of water, dried over $NaSO_4$ and evaporated to dryness.

Phenolic fractions obtained in this way were stored at 0° until 100 l. of urine had been processed. They were then combined and treated at room temperature for 17 hr. with 5 g. of trimethylammonium hydrazide chloride in 50 ml. of ethanol and 10 ml. of acetic acid. The ketonic–phenolic fraction obtained from the reaction mixture in the usual way weighed 284 mg., and by carrying out an analytical partition chromatogram on one-thousandth of the total amount it was shown to contain 9·7 mg. of oestrone and 63·4 mg. of KC-5.

The main bulk of the phenolic–ketonic fraction was chromatographed on a large column (2 cm. diam.) with 120 g. of Celite and the solvent system 70% (v/v) methanol in water–20% (v/v) *n*-hexane in benzene. Thirty successive 40 ml. fractions of the eluate were collected and evaporated to dryness, and Kober reactions carried out on small portions of each. Fractions 19–22, which contained the KC-5, were combined, yielding 55·3 mg. of a slightly pigmented solid which was found to contain 45·5 mg. of Kober chromogen. By washing twice with methanol chilled to −20° a white solid (35·0 mg.) was obtained. After this had been combined with 15·7 mg. of similarly prepared material from another batch of urine it was recrystallized from methanol at −20°, yielding 9·2 mg. of 'Grade I KC-5'. After drying for 2 hr. at 100° *in vacuo* the product had: m.p. 236·5–238·5°; $[\alpha]_D^{15}$, +143° (*c*, 0·254% in ethanol); ϵ_{286}, 2238 in ethanol (Fig. 3). (Found: C, 75·5; H, 7·9. Calc. for $C_{18}H_{22}O_3$: C, 75·5; H, 7·8%.)

By further treatment of the material obtained by evaporation of the methanol washings and mother liquors by rewashing with chilled methanol, 24·6 mg. of 'Grade II KC-5', m.p. 235–238°, and 15·3 mg. of 'Grade III KC-5', m.p. 234–238°, were obtained.

Properties and characterization of KC-5

Kober reaction on KC-5 and 16-oxooestradiol-17β: variation in time of heating in first stage of reaction. The technique used in this experiment was the same as that described by Marrian & Bauld (1955) for a similar experiment with 16-*epi*oestriol (KC-4) and oestriol. For the Kober reactions on KC-5, 10 μg. of the latter (Grade I) was used in each tube, and, for those on 16-oxooestradiol-17β, 11 μg. was used. The curves showing the relationship between optical density at 512·5 mμ. [corrected by using the Allen (1950) formula] are shown in Fig. 2.

Reaction with conc. H_2SO_4 *on KC-5 and 16-oxooestradiol-17β.* To each of two tubes containing respectively 100 μg. of KC-5 (grade I) and 96 μg. of 16-oxooestradiol-17β was added 4 ml. of conc. H_2SO_4, and the mixtures were allowed

to remain at 24° for 2 hr. with occasional shaking. Absorption spectra over the range 230–530 mμ. were determined on both solutions (Fig. 3). It is noteworthy that on adding the H_2SO_4 to the KC-5 an intense red colour developed immediately. This was not observed with 16-oxooestradiol-17β.

KC-5 diacetate. KC-5 (6·7 mg. of grade I + 8·4 mg. of grade III) was treated at room temperature for 24 hr. with 0·5 ml. of acetic anhydride and 0·5 ml. of anhydrous pyridine. After the addition of ice and water, the crude acetate was filtered off, washed thoroughly with water, dried, and crystallized once from a mixture of ethyl acetate and *n*-hexane. The product melted at 166–167°, and the mixed m.p. with 16-oxooestradiol-17β diacetate (m.p. 132·5–134°) was 120–142°. After drying for 1 hr. at 80° *in vacuo* the product had $[\alpha]_D^{13}$, +156° (*c*, 0·301% in ethanol). (Found: C, 71·2; H, 6·8. Calc. for $C_{22}H_{26}O_5$: C, 71·3; H, 7·1%.)

Sodium borohydride reduction of KC-5. To a solution of 20 mg. of KC-5 (Grade II) in 10 ml. of methanol was added 15 mg. of sodium borohydride. After standing at room temperature for 40 min. a further 10 mg. of sodium borohydride was added and the mixture allowed to stand for a further 60 min. About half of the methanol was evaporated off under reduced pressure in a warm-water bath, and after dilution with 100 ml. of water and acidification with 4 ml. of 10N-HCl the mixture was extracted thoroughly with ether. The ethereal extract was washed with aqueous $NaHCO_3$ and with water, and evaporated to dryness. The product was treated at room temperature for 17 hr. with 250 mg. of trimethylammonium hydrazide chloride in 5 ml. of ethanol and 5 ml. of acetic acid, and the non-ketonic material was obtained in the usual way by ether extraction after dilution with water and partial neutralization with NaOH. The non-ketonic reduction product weighed 20·2 mg.

One five-hundredth of the product was chromatographed in the system 70% (v/v) methanol in water–ethylene dichloride, and the results indicated the presence in the total product of about 7·9 mg. of a less 'polar' Kober chromogen and about 9·6 mg. of a more 'polar' one. For comparison a mixture of 15·5 μg. of 16-*epi*oestriol and 16·3 μg. of oestriol were chromatographed in the same system (Fig. 4).

The main bulk of the non-ketonic reduction product was separated into its two components on a large partition column with 120 g. of Celite and the same solvent system. The less and more 'polar' fractions obtained weighed 6·4 and 7·8 mg. respectively.

After one crystallization from methanol–benzene the less 'polar' product melted at 273–276°, and the mixed m.p. with 16-*epi*oestriol (m.p. 284–285°) was 274–281°. The remainder of the recrystallized material, together with that obtained by evaporation of the mother liquor, was acetylated in the usual way with acetic anhydride and pyridine at room temperature. The product, after one crystallization from *n*-hexane, melted at 151–152° and the mixed m.p. with 16-*epi*oestriol triacetate (m.p. 151–153°) was 150–152°. The identification of the less 'polar' reduction product of KC-5 as 16-*epi*oestriol was therefore considered to be satisfactory.

The more 'polar' reduction product melted at 277–279° after one crystallization from methanol–benzene, and the mixed m.p. with oestriol (m.p. 279–280°) was 277–280°.

DISCUSSION

The average yield of KC-5 found in pooled late-pregnancy urine, as determined by analytical partition chromatograms on the ketonic–phenolic fractions, was 0·5 mg./l.; and, since the extraction and fractionation processes employed were certainly not quantitative, it is probable that the average concentration of KC-5 actually present in urine is considerably higher. It is clear, therefore, that this newly discovered α-ketolic Kober chromogen is an oestrogen metabolite of considerable quantitative importance. Although it is present in late-pregnancy urine in such comparatively large amounts, it is hardly surprising, in view of its considerable lability to certain of the processes commonly employed in urine extraction and fractionation, that it has not hitherto been isolated. Rather it is surprising that it was detected in urine extracts by Watson & Marrian (1955), and also, presumably by Pincus & Pearlman (1943) and by Migeon (1953), in experiments in which conditions for its survival must have been far from optimum.

Although it is probable that the material isolated was not a pure substance, convincing evidence has been obtained which shows that the principal component in it must have been either 16α-hydroxyoestrone or 16β-hydroxyoestrone. While other evidence, which has already been discussed, is consistent with the view that the isolated KC-5 was composed of 16α-hydroxyoestrone and some artifactually produced 16-oxooestradiol-17β, the results of the present investigation indicate that 16-oxooestradiol-17β is probably not present as such in urine. Accordingly the speculative suggestion advanced by Marrian & Bauld (1955) that 16-oxooestradiol-17β might be the common metabolic precursor of oestriol and 16-*epi*oestriol receives no support.

The view that the principal component in KC-5 is 16α-hydroxyoestrone rather than 16β-hydroxyoestrone can be supported on biogenetic grounds. It now seems reasonable to suppose that both of these compounds may be formed metabolically from oestrone by 16α- and 16β-hydroxylation, and that they are intermediates in the 'hydration' of oestrone to oestriol and 16-*epi*oestriol respectively. Since the concentration of oestriol in late-pregnancy urine is about 60 times that of 16-*epi*oestriol, it might be assumed that the concentration of the immediate precursor of oestriol would be much greater than that of the immediate precursor of 16-*epi*oestriol.

SUMMARY

1. An α-ketolic Kober chromogen (KC-5) has been isolated from the urine of pregnant women.

2. The evidence obtained shows that the principal component in KC-5 was either 16α-hydroxyoestrone or 16β-hydroxyoestrone. Reasons for favouring the first alternative are given.

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The Occurrence of *n*-Heptadecanoic Acid (Margaric Acid) in Unhydrogenated Mutton Fat

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When margaric acid was formerly isolated from mutton fat (Hansen, Shorland & Cooke, 1954) hydrogenation was employed in the method adopted. Accordingly it was not established whether this acid occurred as such in the natural fat, or whether it resulted (in part or in whole) from hydrogenation of either a C_{17} unsaturated acid or of a *cyclo*propane ring (cf. Hofmann & Lucas, 1950; Hofmann, Lucas & Sax, 1952). As Δ^9-heptadecenoic acid has been identified in lamb-caul fat (Shorland & Jessop, 1955) its presence in mutton fat is expected, and this, on hydrogenation, could account for at least part of the margaric acid present. On the other hand, *cyclo*propane rings have not been shown to occur in natural glycerides, although, as reported by Hofmann *et al.* (1952), they are present in the lipids of certain species of *Lactobaccilus*, and on hydrogenation they yield a methyl-branched-chain acid and a *normal* odd-numbered fatty acid.

In the work now reported a sample of external carcass fat of sheep has been investigated by methods which did not include hydrogenation, and it has been found to contain appreciable quantities of margaric acid.

EXPERIMENTAL

A sample of external carcass fat of sheep (G/99; 31·2 kg.) was steam-rendered, saponified, and converted into fatty acids. The fatty acids were steam-distilled (see McInnes, Hansen & Jessop, 1956), and the steam-non-volatile acids (21·58 kg.) were converted into methyl esters (20·47 kg.) and distilled *in vacuo* in a fractionating column (approx. 180 cm. × 12 cm.) packed with 0·4–0·2 mm. stainless-steel packing of the type described by Lecky & Ewell (1940). Six fractions and a residue resulted, the fourth fraction (F4; 4285 g., saponification equiv. 288·6, iodine value 51·1, m.p. approx. 15·0°) being relevant to this paper. Fraction F4 was refractionated *in vacuo*, a 490 cm. × 3·8 cm. stainless-steel column packed with 3–4 mm. diameter single-turn glass helices being used. The first fraction distilled (F4, 1; 376 g., saponification equiv. 276·8, iodine value 18·8) was then refractionated in column G (described by Shorland, Gerson & Hansen, 1955), yielding 11 fractions (F4, 1, 1–F4, 1, 11) and a residue (F4, 1, R; 37·40 g., saponification equiv. 296·1, iodine value 58·8, m.p. 15·5–16·0°). Fraction F4, 1, R (34·7 g.) was then refractionated in column E (Shorland, 1952) into 13 fractions (F4, 1, R1–F4, 1, R13) and a residue (F4, 1, RR; see Table 1).

Fractions F4, 1, R1–F4, 1, R5 were combined, denoted X1, and as acids submitted to a series of low-temperature

Table 1. *Fractional distillation of methyl ester fraction F4, 1, R (34·7 g.)*

Fraction	Wt. (g.)	M.p. of ester	Saponification equiv.	Iodine value (Wijs)	
F4, 1, R1	1·73	22·0–23·1°	290·5	33·0	
F4, 1, R2	1·97	23·2–25·0	289·7	25·4	
F4, 1, R3	1·49	22·8–24·8	290·7	27·0	X1
F4, 1, R4	0·66	20·0–24·8	290·3	32·7	
F4, 1, R5	2·93	22·3–23·4	291·0	31·9	
F4, 1, R6	4·12	15·5–18·0	293·6	53·5	X4
F4, 1, R7	3·39	−21·0–16·0	294·6	62·6	
F4, 1, R8	3·00	−19·8–14·0	295·4	68·8	
F4, 1, R9	3·25	−17·0–14·3	296·4	72·6	
F4, 1, R10	3·07	−19·0–10·0	297·6	77·9	
F4, 1, R11	2·46	−17·0–15·0	298·0	76·7	
F4, 1, R12	2·51	−17·0–19·5	298·5	75·3	
F4, 1, R13	1·80	−17·0–23·5	299·1	68·5	
F4, 1, RR	2·07	—	451·7	—	

Table 2. *Low-temperature crystallization of fatty acid fraction X1*

Each fraction was crystallized at −40° from 40 vol. of the appropriate solvent.

			Soluble			Insoluble		
Fraction	Wt. (g.)	Solvent	Fraction	Wt. (g.)	M.p.	Fraction	Wt. (g.)	M.p.
X1	7·53	Acetone	X1L	2·75	5·0–6·2°	X1S	4·78	60·8–61·2°
X1S	4·78	Acetone	X1SL	0·11	35·0–36·3	X1SS	4·51	61·2–61·6
X1SS	4·51	Ether	X1S2L	0·16	57·0–58·0	X1S2S	4·33	61·4–61·8
X1S2S	4·33	Ether	X1S3L	0·15	59·2–59·9	X1S3S	4·17	61·8–62·0
X1S3S	4·17	Acetone	X1S4L	0·02	51·8–52·0	X1S4S	4·16	61·2–61·7

crystallizations (see Table 2), finally yielding fraction X1S4S (4·16 g.) with the following properties, which corresponded to those of margaric acid: m.p. 61·2–61·7° (reported m.p.'s: 61·3°, Francis & Piper, 1939; 60·3°, Weitkamp, Smiljanic & Rothman, 1947); when mixed with an equal quantity of pure margaric acid (m.p. 61·3–61·4°) it gave a mixed m.p. of 61·3–61·6°; iodine value 0·0; X-ray long spacing 40·4±0·5 Å [reported values for *n*-heptadecanoic acid: 40·45 Å (Francis & Piper, 1939); 40·05 Å (Slagle & Ott, 1933); 40·3 Å (Stenhagen & von Sydow, 1953)]; n_D^{70} 1·4328 [reported value: n_D^{70} 1·4324 (Dorinson, McCorkle & Ralston, 1942)]. (Found: C, 75·7; H, 12·4%; saponification equiv. 270·6. Calc. for $C_{17}H_{34}O_2$: C, 75·5; H, 12·7%; saponification equiv. 270·4.) Methyl ester: m.p. 29·8–30·3° [reported value: m.p. 29·7° (Francis & Piper, 1939)]; n_D^{40} 1·4352.

A further fraction X4S6S (1·18 g.) was separated by similar low-temperature crystallization from F4, 1, R6 (Table 1). Its properties were as follows: m.p. 61·2–61·5°, saponification equiv. 271·3, X-ray long spacing 39·8±0·5 Å.

As is characteristic of *normal* saturated odd-numbered acids, both fractions X1S4S and X4S6S readily shrank from the walls of their glass containers when allowed to cool from the melt. Melting points were determined in closed capillaries and are uncorrected. The combustion analysis was made by Dr A. D. Campbell, University of Otago, New Zealand. X-ray measurements were made with a Philips Geiger X-ray spectrometer, manganese-filtered iron Kα radiation being used. For the X-ray determination the sample was prepared by evaporation from benzene.

DISCUSSION

The physical and chemical properties of fraction X1S4S establish it as the straight-chain C_{17} saturated acid, *n*-heptadecanoic acid. The occurrence of *normal* saturated odd-numbered fatty acids in natural fats was first established by Jantzen & Witgert (1939), who identified minute traces of *n*-nonanoic, *n*-undecanoic and *n*-tridecanoic acids in coconut oil. Nobori (1942) later confirmed the presence of *n*-undecanoic and *n*-tridecanoic acids in coconut oil. Margaric acid, however, was first obtained from a natural source by Weitkamp *et al.* (1947), who isolated it, together with other *normal* saturated and unsaturated odd-numbered fatty acids, from the free-fatty acid fraction of human-hair fat. Subsequently it was found in hydrogenated mutton fat and ox fat (Hansen *et al.* 1954, 1957) and in shark-liver oil (Morice & Shorland, 1955). In the latter investigation, as in the one now reported, hydrogenation was not employed.

SUMMARY

n-Heptadecanoic acid (margaric acid) has been isolated from unhydrogenated mutton fat.

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Glucose Metabolism in *Candida* Species

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The information available concerning chemical processes in *Candida* species is somewhat limited. In particular, their behaviour towards glucose does not seem to have been studied in detail. Recently, Lodder & Kreger-van Rij (1952) have brought all species of the genus of asporogenous yeast-like fungi known as *Mycoderma*, into the genus *Candida* under the name *Candida mycoderma* (Reess) Lodder & Kreger-van Rij. For the purposes of our inquiry we obtained from the Delft Collection 12 authentic cultures of *Candida* species, including representatives of the former genus *Mycoderma*. In addition, seven strains were selected from a number of cultures of *C. mycoderma*, isolated at Manchester and described by Walker & Wiles (1952).

The behaviour of these 19 organisms in defined media which contained glucose as sole source of carbon was then studied.

EXPERIMENTAL AND RESULTS

Particulars of the strains. Cultures received from the Centralbureau voor Schimmelcultures, Delft, Holland were labelled: (1) *Candida lipolytica* (Harrison) Diddens & Lodder; (2) *Pseudomonilia albomarginata* Geiger; (3) *Candida monosa* (Kluyver) Diddens & Lodder; (4) *Candida krusei* (A. Cast.) Berkhout (= *Mycoderma bordetii* Kuff); (5) *C. krusei* (A. Cast.) Berkhout (= *Mycoderma chevalieri* Guill); (6) and (7) other strains of *C. krusei*; (8) *Candida rugosa* (Anderson) Diddens & Lodder; (9) *Mycoderma lafarii* Janke; (10) *Mycoderma tannica* Asai; (11) *Mycoderma valida* Leberle; (12) *Mycoderma cerevisiae* Desmazières strain *gallica* (Leberle). The seven strains of *Candida mycoderma* which had been isolated at Manchester were designated each by the letter L followed by a number. Stock cultures were maintained on malt-wort agar containing 1% (v/v) of yeast autolysate. In view of the new classification of yeasts by Lodder & Kreger-van Rij (1952) cultures 3–7 are possibly related and cultures 9–12 also are possibly related.

Media and procedure. All the cultures, with the exception of two of the strains of *C. mycoderma*, developed strongly and formed acid in medium *A*, which consisted of: 2·5 g. of $(NH_4)_2HPO_4$, 2 g. of KH_2PO_4, 1 g. of $MgSO_4,7H_2O$, 0·01 g. of $FeSO_4,7H_2O$, 100 g. of glucose, water to 1 l.; the pH value was adjusted to 6·0. The two remaining strains showed only weak growth in this medium but developed well in medium *B*, which contained: 3 g. of $(NH_4)_2SO_4$, 3 g. of KH_2PO_4, 2 g. of $MgSO_4,7H_2O$, 50 g. of glucose, water to 1 l.; the pH value was adjusted to 6·5. Qualitative trials showed that although in these media all nineteen organisms produced pyruvic acid and ethanol from glucose, accumulation of ethanol was very low in cultures of the majority of the strains of *C. mycoderma* (L series). The latter, except in strains L4 and L6, had very weak fermentative capacities. Oxidative ability in the L series was fairly well developed, gluconic, lactic and acetic acids being detected as metabolites. In contrast to *C. mycoderma* the strains of the other species of *Candida*, particularly those of *C. krusei*, were more strongly fermentative and they also exhibited oxidative activities which varied in degree according to the strain, but such oxidative powers were not compared quantitatively with those shown by the strains of *C. mycoderma*.

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Three strains of *C. krusei* (4–6) and one strain of *C. mycoderma* (L 4) were tested for ability to reproduce over a period of time in serial culture in the purely mineral media with glucose as sole source of carbon and energy. They were subcultured with one platinum loopful (3 mm. diameter) into 30 ml. of medium, at weekly intervals, with incubation at 25°. After the sixth serial transfer two of the strains of *C. krusei* grew as vigorously as they had done at their first subculture and the third strain showed a more moderate development. The strain of *C. mycoderma* showed weak development. In all four cases the metabolism solution of the sixth subculture gave positive tests for pyruvic acid, which was detected by the method of Lu (1939) and also by the test described by Caron & Raquet (1942). These reactions were not equally strong but all were quite definite. For purposes of the experiments, the cultures were grown in sets of five 1 l. conical flasks, each charged with 250 ml. of medium *A* or *B*. These flasks had necks of 50 mm. internal diameter and the depth of medium was 30 mm. In a few experiments 250 ml. flasks each charged with 50 ml. of medium were employed. After sterilization, inoculation of each flask was performed with 0·1 ml. of a young culture, which had been developed by inoculation from a malt wort-agar slope into glucose–mineral salts medium of the same composition as that with which the flasks were charged.

Preliminary experiments. The qualitative tests for pyruvic acid were confirmed by isolating it as its *p*-nitrophenylhydrazone and 2:4-dinitrophenylhydrazone, which after purification had m.p. 220° and 218° respectively, undepressed by admixture with the authentic substances. Pure specimens of the 2:4-dinitrophenylhydrazone prepared from cultures of twelve of the strains were combined and the mixture was analysed. (Found: N, 20·7. Calc. for $C_9H_8O_8N_4$: N, 20·9%.) Ethanol was confirmed by oxidation to acetaldehyde, which was characterized as the 2:4-dinitrophenylhydrazone, m.p. 162°. The largest yields of pyruvic acid were given by *C. monosa*, the three strains of *C. krusei* numbered (4), (5) and (6) and strains L 3 and L 4 of *C. mycoderma*. Appreciable yields of pyruvic acid were obtained also from cultures of *Mycoderma valida*, *M. lafarii* and *M. cerevisiae*, but these strains did not form so much ethanol as was obtained from comparable cultures of *C. monosa* and *C. krusei*.

Quantitative studies

Candida krusei (culture no. 5) in medium *A* and *C. mycoderma* (strain L 4) in medium *B* were chosen for detailed studies because the former could set up a vigorous alcoholic fermentation, whereas L 4 afforded ethanol in traces only. Glucose was estimated by the method of Willstätter & Schüdel (1918), allowance being made for the pyruvic acid present. The latter was estimated by the method of Case (1932) and ethanol by oxidation with potassium dichromate (Janke & Kropacsy, 1935). The yields shown in the tables are based on the analysis of 100 ml. of fermented medium in each case.

Estimation of accumulated pyruvic acid. Trials were carried out for the purpose of following the course of pyruvic acid formation in cultures of no. 5 and L 4 (Tables 1 and 2). In both cases accumulation of pyruvic acid in the metabolism solution was followed by its decline to nil. The fact that in neither instance did this behaviour fall into line with the

Table 1. *Formation of pyruvic acid by* Candida mycoderma *L 4, in a medium containing 5 g. of glucose/100 ml.*

Days	Glucose utilized (g.)	Titratable acidity (ml. of 0·1 N-NaOH)			Pyruvic acid (mg.)
		Total	Pyruvic acid, by estimation	Difference, by calculation	
5	0·48	25·5	11·1	14·4	98
7	0·80	36·5	12·5	24·0	110
8	1·02	43·2	11·5	31·7	101
11	1·52	62·4	7·2	55·2	63
14	1·80	67·0	4·3	62·7	38
18	1·99	68·0	Nil	68·0	Nil

Table 2. *Formation of pyruvic acid and ethanol by* Candida krusei *no. 5, in a medium containing 10 g. of glucose/100 ml.*

Days	Glucose utilized (g.)	Titratable acidity (ml. of 0·1 N-NaOH)			Pyruvic acid (mg.)	Ethanol (g.)
		Total	Pyruvic acid, by estimation	Difference, by calculation		
2	0·48	8·8	Trace	8·8	Trace	Trace
4	1·40	27·2	7·8	19·4	69	0·31
6	2·96	42·8	12·4	30·4	109	0·88
7	4·70	59·8	14·1	45·7	124	1·36
8	7·73	70·4	11·4	59·0	100	2·39
9	9·34	52·2	7·7	44·5	68	2·91
11	10·00	45·8	1·4	44·4	12	2·80
14	—	45·0	Nil	45·0	Nil	1·52
27	—	51·0	Nil	51·0	Nil	Trace

figures for titratable acidity shows that in each of these media another acid (or acids) was formed, in addition to pyruvic acid.

The quantity of ethanol formed by no. 5 increased up to a certain limit and then, when other suitable carbon sources were lacking, it decreased, presumably being metabolized by the organism. The residual titratable acid in cultures both of no. 5 and L 4, however, persisted for a long period of time. An account of the acids other than pyruvic acid is reserved for a further communication.

Experiments with nitrates as sources of nitrogen. Wirth & Nord (1942) and Nord & Sciarini (1944) observed accumulation of pyruvic acid in cultures of the mould *Fusarium lini* Bolley on media containing a hexose or a pentose as carbon source. They noted that whereas only traces of pyruvic acid were detectable when $(NH_4)_2SO_4$ was the source of nitrogen its substitution by KNO_3 led to a marked increase in the amount of pyruvic acid accumulating. They considered that this increase might have been caused by nitrite ions (formed by reduction of nitrate) exercising an inhibitory influence on the carboxylase system. In support of this view they obtained positive tests for nitrite in cultures in which the source of nitrogen had been nitrate, and they followed the reduction of the latter to the stage of hydroxylamine. In our experiments, when KNO_3 or $NaNO_3$ was tried as nitrogen source there was very little growth of either no. 5 or L 4, and although tests for pyruvic acid on samples from some of these cultures were positive the quantities present were very small indeed. In no case could the presence of nitrite be detected, although use was made of the very sensitive method of Griess as modified by Blom (1926). When small quantities of KNO_3 were added to the usual media in which $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$ served as source of nitrogen, the yield of pyruvic acid was not raised and nitrite was not detected subsequently in the metabolism solution.

Effects of addition of sodium nitrite to the media. Trials were made to ascertain the effects produced by addition of $NaNO_2$ to the culture media normally used for no. 5 and L 4, and in Table 3 are shown the results obtained with no. 5 on medium *A* to which nitrite was added before inoculation. It is evident that the added nitrite reduced both the rate of utilization of glucose and the amount of pyruvic acid formed. In L 4, addition of nitrite before inoculation strongly retarded growth and lowered the yield of pyruvic acid. Thus the control (no nitrite) produced 41 mg. of pyruvic acid, whereas in the presence of $NaNO_2$ (5 mg./100 ml.) the yield was 6 mg., and was only 1 mg. when $NaNO_2$ was added at 10 mg./100 ml. In these cases the several extents to which cell proliferation occurred were in the same decreasing order of magnitude as were the yields of pyruvic acid.

Effects of addition of thiamine to the media. The accumulation of pyruvic acid in cultures of no. 5 and L 4 suggested that their cells might be deficient in

Table 3. *Effects of* $NaNO_2$ *on accumulation of pyruvic acid formed by* Candida krusei *no. 5 in media containing* 10 *g. of glucose*/100 *ml.*

	Quantity of $NaNO_2$ added before inoculation								
	Control without $NaNO_2$			5 mg./100 ml.			50 mg./100 ml.		
Days	Glucose utilized (g.)	Acid formed (ml. of 0·1 N-NaOH)	Pyruvic acid (mg.)	Glucose utilized (g.)	Acid formed (ml. of 0·1 N-NaOH)	Pyruvic acid (mg.)	Glucose utilized (g.)	Acid formed (ml. of 0·1 N-NaOH)	Pyruvic acid (mg.)
4	2·81	31·1	148	1·99	16·6	76	1·82	13·1	20
8	7·75	56·2	40	3·27	31·2	34	2·67	19·2	26

Table 4. *Effects of thiamine on the behaviour of* Candida mycoderma *L* 4 *and of* Candida krusei *no. 5 in glucose media*

	L 4					No. 5			
	Control, without thiamine		Thiamine (100 μg./100 ml.) added before inoculation			Control, without thiamine		Thiamine (100 μg./100 ml.) added before inoculation	
Days	Glucose utilized (g.)	Pyruvic acid (mg.)	Glucose utilized (g.)	Pyruvic acid (mg.)	Days	Glucose utilized (g.)	Pyruvic acid (mg.)	Glucose utilized (g.)	Pyruvic acid (mg.)
5	0·50	98	1·20	Trace	4	2·29	113	2·02	9
8	1·08	105	1·54	3	7	3·23	170	4·46	10
11	1·58	60	1·74	Trace	11	6·99	134	9·68	Trace
14	1·85	36	—	—	14	9·95	19	10·0	Nil

carboxylase (cf. Barron, 1943). The effects of thiamine hydrochloride on cultures of no. 5 and of L 4 were therefore studied. Addition of this vitamin as the hydrochloride (100 μg./100 ml.), before inoculation, stimulated growth; in both cases proliferation commenced earlier and resulted in greater quantities of cells as compared with the unsupplemented controls. The quantities of pyruvic acid which accumulated fell, however, to very low values (Table 4), demonstrating that the added thiamine had caused utilization of pyruvic acid at a rate almost as great as that at which it was being formed. Similar effects were observed by Wirth & Nord (1942) after addition of thiamine to cultures of *F. lini* Bolley, such addition nullifying the influence of the nitrite ions produced in the cultures. When thiamine hydrochloride (100 μg./100 ml.) was added to cultures of no. 5, 5 days after inoculation, when some pyruvic acid had already accumulated, the rate of fermentation was enhanced and a higher yield of ethanol was obtained, as compared with the control (Table 5).

Effects of addition of sodium arsenite to the media. Arsenite is known to exercise an inhibitory influence on the oxidative decarboxylation of certain α-keto acids which occur as intermediates in biological systems (Krebs & Johnson, 1937). A concentration of 0·02 M sodium arsenite in medium *A* completely prevented the growth of no. 5, and at 0·004 M it just allowed the formation of a faint surface film. At 0·001 M the inhibitory effect was negligible. The effects produced by addition of arsenite (0·002 M) to cultures of no. 5 at 5 days after inoculation are shown in Table 6.

In the presence of arsenite (0·002 M) less glucose was utilized, and pyruvic acid was present in the medium over a longer period of time than was the case in the control; also a markedly higher yield of ethanol was obtained from the medium containing arsenite.

DISCUSSION

The selection of organisms belonging to the genus *Candida* used in this inquiry includes types regarded as virtually non-fermentative, in addition to those capable of setting up comparatively vigorous alcoholic fermentation. All the strains have been found to accumulate pyruvic acid to a greater or less extent when grown in media containing solely inorganic salts and glucose, and in this respect they resemble certain *Fusarium* species (Nord & Mull, 1945). However, there is a marked difference in the behaviour of the two groups of organisms, since species of *Fusarium* accumulate pyruvic acid in cultures containing nitrate as source of nitrogen, whereas species of *Candida* cannot effectively use potassium nitrate for growth and they accumulate pyruvic acid only when the source of nitrogen is a suitable ammonium salt. According to Wirth & Nord (1942), and to Nord & Sciarini (1944), pyruvic acid was caused to accumulate in the culture media of their *Fusarium* species because the action of the carboxylase of this mould was impeded by the nitrite formed by reduction of nitrate in the

Table 5. *Effects of thiamine on the behaviour of* Candida krusei *no. 5 in a medium containing* 10 *g. of glucose*/100 *ml.*

	Control (without thiamine)			Thiamine (100 μg./100 ml.) added on 5th day of incubation		
Days	Glucose utilized (g.)	Pyruvic acid (mg.)	Ethanol (g.)	Glucose utilized (g.)	Pyruvic acid (mg.)	Ethanol (g.)
5	2·53	120	0·189	2·61	129	0·223
7	3·05	175	0·516	4·40	29	1·550
10	4·48	148	1·420	9·78	3	3·120
18	9·91	Trace	1·730	10·00	Nil	0·790

Table 6. *Effects of sodium arsenite on the behaviour of* Candida krusei *no. 5 in a medium containing* 10 *g. of glucose*/100 *ml.*

	Control, without sodium arsenite				Sodium arsenite (0·002 M) added on 5th day of incubation			
Days	Glucose utilized (g.)	Acid formed (ml. of 0·1 N-NaOH)	Pyruvic acid (mg.)	Ethanol (g.)	Glucose utilized (g.)	Acid formed (ml. of 0·1 N-NaOH)	Pyruvic acid (mg.)	Ethanol (g.)
5	2·53	22·0	120	0·189	2·49	27·0	137	0·194
7	3·02	38·5	175	0·516	2·79	27·0	129	0·397
10	4·48	50·0	148	1·420	3·27	31·0	93	0·762
18	9·91	42·5	Nil	1·730	5·64	55·5	33	2·310

medium. Since the *Candida* species, on the other hand, accumulated pyruvic acid in absence of nitrite and there was no other substance present known to be an inhibitor of carboxylase, it would seem probable that when *Candida* species are placed in a glucose–inorganic salts medium they are unable to synthesize thiamine at a rate sufficient for optimum development. That their ability to synthesize thiamine in such a medium varies in degree from one species to another was shown by the results of our tests carried out on cultures maintained in serial transfer (see Experimental and Results, paragraph on *Media and procedure*).

In those experiments in which arsenite was added to cultures of *C. krusei* (no. 5) there resulted a slowing down of the rate at which pyruvic acid was metabolized, but this did not serve to increase the quantity of accumulated pyruvic acid. It would seem probable that under normal conditions pyruvic acid is broken down by this organism partly by simple decarboxylation and partly by oxidative decarboxylation, but in the presence of arsenite the latter change is impeded, with the result that simple decarboxylation can occur to a greater extent, thus giving rise to a higher percentage yield of ethanol as compared with the arsenite-free control. Thus after 18 days (Table 6) the medium containing arsenite yielded 2·31 % of ethanol and the control afforded only 1·73 % of ethanol.

SUMMARY

1. Nineteen cultures representing not less than six species of *Candida* grew well in simple media of inorganic salts and glucose, with either ammonium phosphate or ammonium sulphate as source of nitrogen, and all the strains formed: (*a*) pyruvic acid, (*b*) another organic acid (or acids) and (*c*) ethanol, which in some cases was detected in traces only.

2. *Candida* species were unable to utilize nitrates as source of nitrogen, and were restricted in their growth and acid-forming capacity by addition of nitrite to the medium.

3. When thiamine hydrochloride (100 μg./100 ml.) was added to cultures of *C. krusei*, which normally produces an appreciably large yield of ethanol from glucose, the rate of utilization of glucose was increased, accumulation of pyruvic acid was prevented and the yield of ethanol was greater than that afforded by the control without added thiamine. Addition of thiamine hydrochloride (100 μg./100 ml.) to cultures of the very weakly fermentative *C. mycoderma* caused an increase in the rate of utilization of glucose and prevented accumulation of pyruvic acid, but did not promote formation of ethanol.

4. Addition of sodium arsenite (0·002 M) to cultures of *C. krusei* caused the rate of utilization of glucose to fall and the yield of ethanol to be substantially increased.

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*meso*Inositol in the Lens of Mammalian Eyes

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This work was undertaken for two reasons. First to confirm and extend the findings of Krause & Weekers (1938), who reported a high level of free inositol in the lens of three- to four-year-old cattle, considerably higher than that in other tissues of the eye. They used the method of Young (1934) and found the following values (in mg./100 g. of fresh tissue): lens, 126–177; optic nerve, 89–111; cornea, 6–8; iris, 22–35; retina, 13–17; choroid, 29–35; sclera, 10–14; conjunctiva, 6; traces were found in the vitreous humour. The aqueous humour was not analysed. Secondly, the nature of a considerable fraction of the trichloroacetic acid-soluble phosphorus compounds in the lens is unknown (Pirie, van Heyningen & Flanders, 1955). It seemed possible that some of the phosphorus in this fraction might be in the form of inositol phosphates. The only acid-soluble phosphorylated inositol previously found in

normal mammalian tissues is phytic acid (inositol hexaphosphate), which occurs in nucleated erythrocytes (Rapoport, 1940). However, the search for such compounds does not appear to have been extensive.

Inositol has been found in the phosphatide fraction of the lipids of many animal tissues (Folch & Woolley, 1942; Folch, 1949; Hawthorne, 1955). The lens contains a small amount of phospholipid (about 200 mg./100 g. of cattle lens, aged one year; Krause, 1935), and consequently the protein plus lipid fraction of the lens has been examined for evidence of combined inositol.

METHODS

Preparation of lens extracts. Cattle, pig and sheep eyes were removed from the animals as soon as possible after death and brought to the laboratory on ice. They were used at once, or stored in the refrigerator at 0–4° for not more than an hour before use. The lenses from the eyes of laboratory animals were removed and extracted immediately after death. Lenses from human eyes were obtained *post mortem*. Two types of extract were made: (i) Trichloroacetic acid. The lens was ground in a known volume of ice-cold 5% (w/v) trichloroacetic acid, kept in the refrigerator at 0–4° for 30 min. and then centrifuged at 7000 *g* for 10 min. The extracts were stored at −15°. The trichloroacetic acid did not interfere with the inositol estimations. (ii) Acetate buffer at 100°. The lens was dropped into a graduated tube containing about ten times its weight of 0·1N acetate buffer, pH 5·2, standing in a boiling-water bath. The lens was thoroughly disintegrated by rubbing it with a glass rod against the side of the tube. After 4 min. the tube was removed and cooled. The contents were made up to a known volume and filtered or centrifuged. The clear extract was stored at −15°.

Hydrolysis of lens extracts and residues. A known volume of extract was boiled under reflux for 6 hr. with an equal volume of conc. HCl (Woolley, 1941). Most of the HCl was removed *in vacuo* and the hydrolysed extract was neutralized, made up to a known volume with water and its inositol content estimated. Any increase in the amount of free inositol in the extract after hydrolysis is assumed to give a measure of the amount of combined inositol originally present, since hydrolysis with acid under these conditions is believed to hydrolyse all forms of phosphorylated inositol (Woolley, 1941).

On some occasions the insoluble residues from the above-mentioned extraction procedures (i) and (ii) were examined for the presence of inositol. After three washings with large volumes of either trichloroacetic acid or acetate buffer they were hydrolysed as described above and the hydrolysates used for the estimations.

Estimation of inositol. This was estimated by microbiological assay with *Saccharomyces carlsbergensis* (American Type Culture Collection no. 4228). The method was based on that of Atkin, Schultz, Williams & Frey (1943) (see Snell, 1954). The basal medium described by these workers was modified by omitting inositol and adding pyridoxine (1 mg./l. of medium). Incubation was for 18–24 hr. at 37° in 18 mm. × 150 mm. tubes containing 2 ml. of medium; the tubes were incubated in a sloped position to increase aeration. Growth was measured turbidimetrically with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex). Inositol could be assayed by this method with an accuracy of ±10%. The method is specific for free *meso*inositol (also called myo-inositol or inositol), the stereoisomer of hexahydroxy*cyclo*hexane, which is most widely distributed in nature (Woolley, 1947).

Estimation of glutathione. The method of Grunert & Phillips (1951) was used on trichloroacetic acid extracts of lens.

Irradiation of lens. The technique was as previously described (Pirie, van Heyningen & Boag, 1953; Pirie *et al.* 1955). The right eye of a seven-week-old rabbit was irradiated with X-rays (800–1000 r.) to produce the first signs of cataract (i.e. opacity) in the lens about 10 weeks later. Thereafter the cataract progressed rapidly. The left eye and lens were unaffected. The rabbits were killed and their lenses analysed at varying intervals after the first sign of cataract. On two occasions the lenses of rabbits with complete X-ray cataracts in the right eye were analysed. These rabbits were irradiated by the same technique, except that the right eye received 1400 r.; one (no. 9, Table 2) was seven weeks old at irradiation and the lens was fully opaque 10 weeks later. The other (no. 10, Table 2) was 26 weeks old at irradiation and the lens took about 30 weeks to become opaque.

RESULTS

Free inositol in the lens

Table 1 gives the values obtained for free inositol in the lens of various species of animal. The lens of each species examined contains inositol, though there appears to be considerable difference in concentration between species. The level of inositol in the lens of the very young animal (under two weeks old in cattle, and during the first week after the eyes are open in rabbits) was always lower than that in the adult animal of the same species (Table 1).

An adult cattle lens was divided into three concentric zones of approximately equal weight as follows. After removal of the capsule, the soft outer fibres (the lens cortex) were gently scraped off with the blunt side of a scalpel blade. The firmer layers beneath (middle zone) were then separated from the hard central core (lens nucleus) by more vigorous scraping. The three zones were analysed for inositol and it was found that the concentration decreased towards the centre of the lens; thus the three zones, lens cortex, middle layer and lens nucleus, contained 336, 307 and 213 mg. of inositol/100 g. of tissue respectively.

Free inositol in the cataractous lens

Table 2 gives the results of comparisons between the free inositol concentration in the normal rabbit lens (left) compared with its cataractous fellow lens (right). Glutathione (GSH) concentrations are included, as decrease in GSH is the earliest known chemical change in cataract development and may

Table 1. *Free inositol in the lens*

Species	Age	Lens wt. (approx.) (g.)	Inositol (each value is from a different animal) (mg./100 g. of lens)
Cattle			
Calf	Probably under 14 days	1·0	106, 128, 137
Adult	Probably 1–2 years	2·0–2·5	250, 298, 312, 343, 365
Rabbit	10 days	0·1	34, 58
	11 days	0·1	65
	13 days	0·1	63
	14 days	0·1	70
	17 days	0·1	32
	8–30 weeks	0·25–0·45	115, 117, 128, 136, 153, 156, 160, 175, 184, 190, 211
	2 years	0·55	106, 107
Pig	Unknown	0·4	315, 452
Rat	7 weeks	0·03	51
	35 weeks	0·05	83
Human	62 years	0·5	414
	69 years	0·3	509
Mouse	Unknown (adult)	0·01	142, 162
Guinea pig	Unknown (adult)	0·07	450, 490
Sheep	Unknown (adult)	1·0	490, 517, 553

Table 2. *Comparison of the concentrations of inositol and glutathione in normal and cataractous lens*

The right lens (R) of rabbits 1–10 was irradiated (see Methods). L, Left lens.

Rabbit		State of right lens*		Glutathione mg./100 g.	Glutathione Difference (%) $\left(\frac{R-L}{L}\times 100\right)$	Inositol mg./100 g.	Inositol Difference (%) $\left(\frac{R-L}{L}\times 100\right)$
Control		(Not irradiated)	R	—	—	195	+5
			L	—		185	
Control		(Not irradiated)	R	—	—	103	−6
			L	—		110	
Litter mates	1	(+)	R	565	−2	190	+9
			L	575		175	
	2	(+)	R	490	−7	175	+9
			L	525		160	
	3	(+)	R	505	−8	180	−14
			L	550		210	
Litter mates	4	(+)	R	415	−6	146	−6
			L	440		156	
	5	+	R	450	−12	107	−16
			L	511		128	
	6	+	R	449	−8	146	+25
			L	489		117	
Litter mates	7	+	R	450	−5	96	−16
			L	470		115	
	8	++	R	410	−20	118	−13
			L	514		136	
	9	+++++	R	99	−79	13	−91
			L	485		153	
	10	+++++	R	—	—	2	−98
			L	—		106	

* (+), Almost normal; +, small cortical opacities; ++, marked opacities; +++++, completely opaque.

be used to assess other changes (Pirie *et al.* 1955). The table shows that nearly all the inositol is lost from a lens with a complete cataract. It is difficult, however, to decide at what stage in the development of cataract the reduction in inositol content begins. Whereas the GSH content of the lens can be measured with an accuracy of ±3–4% (Pirie *et al.* 1955), the limit of accuracy in the measurement of inositol is about ±10%. The table shows that differences of 5 and 6% were found between the inositol content of two pairs of normal lenses; these differences are well within the limit of experimental error of the method of estimation. Of the right lenses showing early cataractous changes, some had a higher and some a lower inositol content than the normal fellow, and there is no evidence of a consistent decrease in inositol concentration. Decrease in GSH concentration was, as usual in X-ray cataract, a consistent finding. However, since the experimental error involved in inositol estimation is much greater than that for GSH, with the result that small differences in concentration cannot be detected, it is not possible to conclude that the GSH content decreases before inositol. Furthermore, for the same reason, it was not considered profitable to examine more X-ray cataracts at an early stage. The only conclusions that can be drawn from the figures are that at some stage after X-irradiation the inositol content of the lens starts to diminish, that by the time the first signs of cataract appear this diminution may or may not have begun, but that by the time the lens is fully opaque the inositol has almost completely vanished.

It can also be seen from Table 2 that variation between the inositol concentration of the normal (left) lenses of litter mates was considerable, though no greater than the variation in glutathione concentration. All three animals of a litter were killed on the same day.

Table 3. *Examination of lens for presence of combined inositol*

See Methods for preparation of extracts; hydrolysis was in 6N-HCl for 6 hr., boiling under reflux. TCA, Trichloroacetic acid.

Species	Material examined	Inositol (mg./100 g. of lens) Before hydrolysis	After hydrolysis
Cattle			
Calf	TCA extract	137	138
	TCA residue	—	1
Adult	TCA extract	343	323
	Acetate buffer extract	365	365
Pig	TCA extract	315	290
	TCA residue	—	3
Rabbit	Acetate buffer extract	184	184
	Acetate buffer residue	—	1

Combined inositol in lens

Table 3 shows that there is no evidence for the presence of combined inositol in acetate buffer or trichloroacetic acid extracts of calf, cattle, pig or rabbit lens. Furthermore, hydrolysis of the insoluble residue after trichloroacetic acid or acetate buffer extraction yielded negligible amounts of inositol, thus showing that inositol is probably not a constituent of the phospholipid of lens.

Inositol in lens after incubation in vitro

A calf lens was immersed in 10 ml. of Krebs–phosphate Ringer containing glucose (1 g./l.) in a wide-necked centrifuge tube (32 mm. diameter). The unstoppered tube was incubated in a water bath at 37° for 5 hr. with occasional gentle shaking. Under these conditions the lens continues to metabolize glucose to form principally lactic acid (Huysmans & Fischer, 1941; Merriam & Kinsey, 1950). Inositol was estimated in the lens and suspension fluid after incubation and was found to be the same (1·21 mg./lens) as in the fellow lens, analysed without incubation (1·27 mg./lens). Only traces (about 0·06 mg.) of inositol leaked out of the lens into the fluid during the 5 hr. incubation.

Inositol in the aqueous humour

Estimations of free inositol in the aqueous humour of the rabbit and the cattle eye gave values of 2 mg./100 ml. and 5 mg./100 ml. respectively. The level of inositol in rabbit blood is 0·8–2·7 mg./100 g. of blood (Campling & Nixon, 1954).

DISCUSSION

The high concentration of free *meso*inositol in the lens has been confirmed (Table 1); in some species it is higher than that recorded for any other animal tissue (Woolley, 1947; Fleury & Balatre, 1947), although an animal secretion, that of the seminal vesicle of the boar, contains a very much greater concentration (2–3 g./100 ml.; Mann, 1954). In the cattle lens the concentration of *meso*inositol is from 14 to 20 mM. This may be compared with the concentration in the cattle lens of three other low-molecular-weight, water-soluble organic compounds, which occur at rather high levels and which are known to be or are suspected to be concerned with its metabolism. They are all retained without loss within lens tissue during several hours of incubation *in vitro* under physiological conditions. These compounds are adenosine triphosphate (ATP), 1 mM (Müller, 1937; Nordmann & Mandel, 1952), glutathione, 5 mM (Dische & Zil, 1951; Kleifeld & Hockwin, 1956) and ascorbic acid, 2 mM (see Bellows, 1944). Thus *meso*inositol is present in a considerably higher concentration than any of

these, but its function in the lens is entirely unknown and has scarcely been considered.

The lens of the rabbit has been examined more thoroughly than that of any other species for changes due to age or cataract formation. *meso*-Inositol, like glutathione (Pirie *et al.* 1955) and probably ascorbic acid (Kinsey, Jackson & Terry, 1945), but unlike ATP (Pirie *et al.* 1955), is at a lower concentration in the lens of the very young rabbit than in the adult rabbit (Table 1). Like glutathione (Pirie *et al.* 1953) and ATP (Pirie *et al.* 1955), it is mostly lost from a rabbit lens made completely cataractous by X-irradiation (Table 2), but limitations in the accuracy of estimation make it impossible to decide at what stage in the formation of cataract its concentration begins to decrease.

SUMMARY

1. Free *meso*inositol is found in the lens of several species of animal, ranging in concentration from about 50 mg./100 g. of lens in the rat to about 500 mg./100 g. of lens in the sheep and the human. This is higher than that recorded for any other animal tissue. It is present in the aqueous humour (rabbit, cattle) at a much lower concentration.

2. The concentration of *meso*inositol in the lens of the very young animal (cattle, rabbit) is lower than that in the adult animal of the same species.

3. There is no evidence of any combined form of *meso*inositol in the lens.

4. *meso*Inositol is virtually absent from the lens of a rabbit made completely opaque by means of X-rays, but limitations in the accuracy of its estimation do not permit conclusions as to the stage of cataract development at which the diminution begins.

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The Conversion of Glucose into Alanine and Glutamic Acid by Rat Liver, Liver Tumour and Kidney *in vivo*

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In studies on the metabolism of protein by tumour-bearing animals there often arises the problem whether a given protein has been synthesized by the tumour or by the normal tissues of the body. It seemed possible that this problem might be resolved by taking advantage of the differences in carbohydrate metabolism between tumour and normal tissues first described by Warburg (1930). He showed that, in general, tumour tissue has a higher rate of aerobic glycolysis than normal tissue. It might, therefore, be expected that the rate of turnover of oxoglutarate in tumour tissue relative to that of pyruvate would be lower in tumour than in normal tissue. As a result the rate of conversion

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of glucose into alanine and glutamic acid might differ in the two types of tissue. Thus if protein synthesis took place in the presence of [^{14}C]glucose the ratio of the radioactivity of glutamic acid to that of alanine in the protein of the two types of tissue might be different.

In this paper an attempt has been made to test this possibility by injecting [^{14}C]glucose into rats bearing liver tumours and determining the radioactivity of the alanine and glutamic acid in the liver and tumour protein after one hour. For the purposes of comparison kidney protein has also been studied.

To enable the conversion of glucose into amino acids in the tissues to be studied in greater detail, the incorporation into the tissue proteins of an essential amino acid which is relatively inert, [^{14}C]lysine, was also followed. Estimations were made of the radioactivity of the free alanine, glutamic acid and lysine in the tissues.

MATERIALS AND METHODS

Animals and production of liver tumours. Tumours were induced in rats by feeding them on a diet containing 0·06% of *p*-dimethylaminoazobenzene as described by Campbell (1955).

Radioactive substances. Uniformly labelled D[^{14}C]-glucose (100 μc≡1·29 mg.) and L[^{14}C]-lysine (100 μc≡2·5 mg.) were supplied by the Radiochemical Centre, Amersham.

Injection of [^{14}C]glucose and [^{14}C]lysine into rats. Three rats, weighing about 350 g. each, whose livers were infiltrated with tumour to varying degrees, were chosen after laparotomy. Thus rat 1 was badly infiltrated, rat 2 less so and in rat 3 the tumours were very small. At the time of injection, rats 1 and 2 had been on a normal diet for 2 months and rat 3 for 6 weeks, after the diet containing *p*-dimethylaminoazobenzene. All three rats were left for 1 week after laparotomy before injection. Each rat, under light ether anaesthesia, was given, by intravenous injection into the femoral vein, 1 ml. of a saline solution containing 20 μc of [^{14}C]glucose (260 μg.) and 5 μc of [^{14}C]lysine (125 μg.). After 60 min. the rats were anaesthetized with ether and each liver was perfused with 40 ml. of warm saline. The required tissues were cooled on ice.

Isolation of tissue proteins. The tumours were dissected free of liver tissue and liver tissue was similarly dissected free of tumour. Samples of liver, tumour and kidney tissue were then homogenized in a Potter-type glass homogenizer in cold saline. The tissue suspensions were centrifuged at 3000 *g* for 15 min. at 5° and the partially cleared supernatants were frozen overnight. The supernatants (*S*) were again centrifuged and filtered through Whatman no. 1 filter paper. The proteins were precipitated by addition of an equal volume of 10% (w/v) trichloroacetic acid and the precipitates (*P*) were heated to remove non-protein substances as described by Zamecnik, Loftfield, Stephenson & Steele (1951).

Isolation of free amino acids from tissues. The supernatant (*S*) obtained after the addition of trichloroacetic acid to the cleared tissue extracts (see above), together with the washings from the first treatment of the precipitated protein with 5% (w/v) trichloroacetic acid, was extracted three times with 3 vol. of ether to remove the acid. The amount of ninhydrin-positive substances present in the aqueous extracts was determined by the procedure of Cocking & Yemm (1954) (see also Campbell, 1955). The liver extract contained 24 mg. of amino acids calculated as alanine, liver tumour 48 mg. and kidney 17 mg.

The amino acids in the extracts were fractionated on columns of Dowex 1 (10% cross-linked, particle size 50 μ., 0·9 cm. diam., 30 cm. long) and Zeo-Karb 225 (W.R. 1·55, particle size 50 μ., 0·9 cm. diam., 100 cm. long; The Permutit Co. Ltd, London, W. 4) (Campbell, Jacobs, Work & Kressman, 1955) according to the method of Hirs, Moore & Stein (1954). With the Dowex 1 column 0·5 M acetic acid was followed by 4 M acetic acid and then N-HCl. With the Zeo-Karb column gradient elution was not used, approximately N-HCl (10% v/v AnalaR HCl) being followed by approximately 2·5 N-HCl (25% v/v AnalaR HCl).

Since the capacity of the ion-exchange columns was limited, only one-half of the tumour extract was fractionated, whereas the whole of the kidney and liver extracts were used.

The fractions from these columns containing the required amino acids were concentrated and examined by paper chromatography, not only for ninhydrin-positive substances, but also for u.v. light-absorbing and -fluorescing substances. The concentrates containing glutamic acid from tumour and kidney were thus shown to be free from other ninhydrin-reacting substances. The concentrate containing glutamic acid from liver was contaminated by another ninhydrin-positive substance so that this fraction was further purified by paper chromatography with Whatman no. 3 paper and butanol–acetic acid–water (4:1:5, by vol.) as eluent (see Campbell, 1955). The fractions containing alanine were contaminated in every case with two other ninhydrin-positive substances and were, therefore, purified by paper chromatography as described for glutamic acid. The fractions containing lysine were always free from other ninhydrin-reacting substances, although the fraction from liver did contain a u.v. light-absorbing substance. For this reason some of the liver fraction was further purified on Whatman no. 3 paper.

Specific radioactivity of free amino acids (F). These determinations were carried out by counting at infinite thinness on 2 cm.^{2} polythene disks as described for alanine by Campbell (1955). The amount of amino acid on each disk was estimated by reaction with ninhydrin. It was found that when the specific activity of an amino acid was expressed as counts/min./mg. at infinite thinness the counts/min./cm.^{2} at infinite thickness could be calculated by multiplying the infinite thinness count by 7·6.

To ensure that the use of Whatman no. 3 paper for the final purification of the amino acids from the tissue extracts did not invalidate the determination of their specific radioactivity a control experiment was carried out with a standard solution of [^{14}C]alanine. The results showed that such a procedure did not significantly change the specific radioactivity of the amino acid.

Isolation of amino acids from proteins (P). Approximately 500 mg. of the tumour and liver proteins (*P*) was hydrolysed in a sealed tube with 10 ml. of 6 N-HCl at 105–110° for 22 hr. After removal of excess acid the amino acids were isolated on columns of Dowex 1 (3 cm. diam., 30 cm. long) and

Zeo-Karb 225 (3 cm. diam., 100 cm. long) as described above. The effluent from the columns was analysed by paper chromatography and the fractions containing glutamic acid, alanine and lysine respectively were pooled. In every case, these fractions were uncontaminated by other ninhydrin-positive substances.

With kidney protein 60 mg. was hydrolysed with 2 ml. of 6N-HCl as above. The amino acids from this hydrolysate were separated on small columns of Dowex 1 and Zeo-Karb 225 as described for the free amino acids.

The individual amino acids were isolated as follows: after removal of acetic acid glutamic acid was cryst. from aqueous ethanol. After removal of HCl alanine was converted into its dinitrophenyl (DNP) derivative according to the method described by Porter (1950). The reaction mixture consisted of 8 ml. of 50% (v/v) aqueous ethanol, 100 mg. of anhydrous Na_2CO_3 and approximately 50 μl. of 1-fluoro-2:4-dinitrobenzene. The mixture was shaken for 2 hr. at room temp. The DNP-alanine was crystallized from a mixture of ether and light petroleum (b.p. 60–80°). Lysine was converted into its di-DNP derivative under the same conditions as for alanine but was crystallized from aqueous formic acid.

Specific radioactivity of amino acids from protein. All amino acids or their derivatives were counted at infinite thickness on either 0·28 cm.^{2} Perspex disks or 1 cm.^{2} polythene disks. The counts obtained for the amino acid derivatives were converted into the free amino acids for alanine and glutamic acid and to the monohydrochloride for lysine.

RESULTS

Radioactivity of amino acids from protein hydrolysates

The weights of the samples of the various tissues used, together with the weight of soluble protein precipitated by trichloroacetic acid obtained from each, are shown in Table 1. Table 2 gives the radioactivity of the amino acids isolated from the proteins precipitated by trichloroacetic acid. The coefficient of variation of these determinations was 3–7%. For the determination of the average radioactivity of the amino acids from liver and tumour equal quantities of protein from each rat were hydrolysed together.

Radioactivity of the free amino acids from the tissues

It is, of course, very much more difficult to isolate samples of pure amino acids from tissue extracts than from protein hydrolysates. Thus, in many cases, fractionation of the tissue extracts on ion-exchange columns gave fractions containing more than one ninhydrin-positive substance. It was often necessary to purify the fractions further by partition chromatography on thick paper which had been extensively washed before use.

Although these methods of purification enabled fractions to be obtained which contained only one ninhydrin-positive substance it was thought that the fractions might contain significant amounts of substances which did not react with ninhydrin. It was for this reason that the chromatograms were scanned with ultraviolet light. In every case except that of the liver lysine the fractions were free of ultraviolet light-absorbing or -fluorescing substances. Further purification of the liver-lysine fraction by paper chromatography showed that the specific activity of the liver lysine was not significantly affected by the presence of the ultraviolet light-absorbing impurity. Small quantities of non-radioactive impurities in the tissue extracts would not be expected to affect the specific radioactivity

Table 1. *Amount of trichloroacetic acid-precipitable protein obtained from supernatants of homogenates of rat tissues*

Tissue wt. represents wet wt. of tissue. All weights in g.

	Liver		Liver tumour		Kidney	
Rat	Tissue	Protein	Tissue	Protein	Tissue	Protein
1	11·2	0·750	22·5	1·240	1·9	0·116
2	10·2	0·640	7·0	0·360	—	—
3	13·2	0·560	—	—	—	—

— Signifies values not determined.

Table 2. *Radioactivity of alanine and glutamic acid isolated from soluble tissue proteins 1 hr. after injection of rats with* [^{14}C]*glucose*

Radioactivity is given in counts/min./cm.^{2} at infinite thickness.

	Liver		Liver tumour		
	Rat 1	Average of rats 1–3	Rat 1	Average of rats 1 and 2	Kidney Rat 1
Alanine	158	132	232	186	46
Glutamic acid	56	50	78	52	41
Ratio alanine/glutamic acid	2·8	2·6	3·0	3·6	1·1

of the amino acids since the latter was determined at infinite thinness.

Since at least 500 μg. of 'pure' amino acid was always obtained it was possible to make several determinations of the specific radioactivity on each sample. The coefficient of variation between the various determinations was usually in the range of 6–9 %. However, where the counts were rather low, as with alanine and glutamic acid from kidney, the coefficient of variation rose to 15 %.

The results obtained from the determination of the specific activity of the free amino acids from the tissues of rat 1 are shown in Table 3 together with the activity of the protein-bound amino acids from the same tissues. It will be seen that in every tissue the F/P ratio for glutamic acid is higher than that for alanine. The difference between the F/P ratio of lysine for the three tissues is also significant.

Nature of the amino acids, peptides and related substances in the tissues

Although it was not the object of the present work to study differences between the free amino acids and peptides in the different tissues, some observations on these are of interest. Thus on Dowex 1 columns taurine was found in all the tissue extracts, but occurred in much higher concentration in those of liver than of tumour or kidney.

The behaviour of glutathione on columns of Dowex 1 was studied with an authentic sample. When the eluent on the column was changed after 95 ml. of effluent from 0·5 M to 4 M acetic acid, glutathione was eluted in a discrete band after a further 60–70 ml. Glutathione was thus easily detected in extracts of liver, whereas only traces were found in kidney extract and it was not detected at all in tumour extracts. This would appear to be a convenient way of isolating small quantities of glutathione. The low concentration of glutathione in liver tumour compared with liver is in agreement with the results of Zamecnik & Stephenson (1949), who estimated the concentration of glutathione in these tissues by an enzymic method.

After eluting the Dowex 1 columns with 4 M acetic acid they were washed with N-HCl and the concentrated effluent was examined by paper chromatography. No more than traces of ninhydrin-reacting substances were present in the tumour or kidney extracts, but the liver extract contained considerable amounts of such substances. When this fraction from liver was hydrolysed, there was a very marked increase in the number and amount of ninhydrin-reacting substances. This suggests the presence of conjugated amino acids in this fraction from liver.

The patterns of amino acids emerging from the columns of Zeo-Karb 225 were remarkably similar for the three tissue extracts. Asparagine was identified in all cases. Alanine was always contaminated by two unknown ninhydrin-reacting substances, the one with a peak just before the alanine peak and the other with a peak just after.

DISCUSSION

Following the demonstration by Warburg (1930) that the rate of aerobic glycolysis in tumours is higher than that usual for non-tumour tissues, it was thought possible that there is a difference in the pathway of oxidation of carbohydrate in the two types of tissue. In particular, the possibility that the tricarboxylic acid cycle might be relatively deficient in tumour as compared with normal tissue has been considered.

Table 3. *Radioactivity of amino acids in the supernatant fraction of homogenates of rat tissues* 1 *hr. after injection of* [^{14}C]*glucose and* [^{14}C]*lysine*

Radioactivity of the amino acids isolated from the protein precipitated with trichloroacetic acid is compared with that of the free amino acids in the extract. Radioactivities have been corrected to counts/min./cm.^{2} at infinite thickness. F/P ratios are expressed as $\pm$S.E.M. derived from the coefficient of variation of the radioactivity determinations of the protein and free amino acids.

Amino acid isolated	Protein amino acid (P)	Free amino acid (F)	Ratio F/P	$\frac{F/P \text{ lysine}}{F/P \text{ amino acid}}$
		Liver		
Alanine	158	2 519	16$\pm$1·6	0·38
Glutamic	58	1 275	22$\pm$2·6	0·28
Lysine monohydrochloride	980	5 978	6·1$\pm$0·54	1·0
		Liver tumour		
Alanine	232	6 396	28$\pm$2·9	0·34
Glutamic	78	3 572	46$\pm$2·6	0·21
Lysine monohydrochloride	1100	10 300	9·4$\pm$0·80	1·0
		Kidney		
Alanine	46	591	13$\pm$1·6	0·27
Glutamic	41	1 244	30$\pm$4·9	0·12
Lysine monohydrochloride	829	2 900	3·5$\pm$0·29	1·0

The very extensive studies which have been carried out on this subject have recently been reviewed by Weinhouse (1955). Although no qualitative differences in the metabolic pathways occurring in tumour and normal tissue have been found, marked differences of a quantitative nature have come to light. Thus Olson (1951) showed that slices of normal liver and liver tumour differed considerably in their metabolism of carbohydrate. It seemed possible that such differences might be reflected in the rate of conversion of glucose into alanine and glutamic acid by these tissues *in vivo*, which in turn might lead to a difference in the ratio of the radioactivity of alanine and glutamic acid when protein was isolated from the tissues after injection of [^{14}C]glucose.

The radioactivity of alanine and glutamic acid isolated from liver, liver tumour and kidney one hour after injection of [^{14}C]glucose is shown in Table 2. It will be seen that while the ratio of the radioactivity of alanine to that of glutamic acid from liver tumour is a little higher than the ratio from liver the differences are not very marked. However, the corresponding ratio for kidney is considerably lower than that of the other two tissues. Although this result shows that [^{14}C]glucose cannot be used to characterize protein synthesized by liver tumour as opposed to that synthesized by liver it provides little information regarding the rate of formation of glutamic acid and alanine from glucose in the tissues. It was for this reason that [^{14}C]lysine was injected with [^{14}C]glucose.

If an animal is injected with a radioactive amino acid, the rate at which this amino acid becomes labelled in the proteins of a given tissue depends on the radioactivity of the injected amino acid in the tissue and the rate of protein synthesis in that tissue. The permeability of the tissue to the injected amino acid and the amount of inactive amino acid with which the radioactive amino acid becomes equilibrated are likely to be the most important factors determining the radioactivity of the injected amino acid in a particular tissue. Thus the ratio of the radioactivity of a free amino acid in a tissue and that of the same acid in the tissue protein following the administration of a radioactive amino acid will be a measure of the rate at which that amino acid becomes incorporated into the tissue protein. In the absence of exchange between free amino acid and protein-bound amino acid the latter will be a measure of the rate of protein synthesis.

Ideally, it would be best to measure the ratio of the free amino acid and protein-bound amino acid radioactivity during the whole course of protein synthesis. In practice this presents difficulties. However, if the activity of the free amino acids is determined before complete equilibration between the amino acid pools in the tissues has taken place it is probable that the result will be indicative of the degree of dilution within the tissue of the injected radioactive amino acid. That complete equilibration of the free amino acids between the tissues had not taken place when the tissues were removed from the rat in the present experiments is indicated by the wide variation in the radioactivity of free lysine in the tissues (Table 3).

It is, of course, theoretically possible to inject so much radioactive amino acid that differences in the sizes of the pools of amino acids in the tissues are eliminated. Such a technique was attempted by Babson & Winnick (1954) by injecting massive doses of leucine into rats, but as Loftfield & Harris (1956) have shown, so much of the tissue amino acids are derived from the breakdown of tissue protein that it is doubtful whether this situation can be achieved in practice.

In the present experiments only 125 μg. of lysine was injected into each 350 g. rat. Since Sassenrath & Greenberg (1954) have shown that even the total plasma of such a rat would contain about 1 mg. of free lysine, it is unlikely that the amount injected in this case would significantly change the amount of free lysine available to the tissues.

From Table 3 it will be seen that the ratio of free tissue lysine to protein lysine one hour after injection of [^{14}C]lysine is in the descending order: kidney, liver, liver tumour. Since the free lysine had a very much higher radioactivity than the protein lysine, we may conclude that the rate of incorporation of amino acids is greatest in kidney, followed by liver and then liver tumour. This conclusion is only strictly valid if the incorporation of amino acid into a homogeneous protein is being studied. In the present experiments a mixture of soluble tissue proteins was used and no doubt these consist of components of differing amino acid composition having different turnover rates. Under these conditions the radioactivity of a particular amino acid at a given time after injection would depend on both turnover rates and the amino acid composition of the various components. Fortunately the concentration of lysine in proteins does not vary very greatly and it is possible that the soluble tissue proteins represent such a heterogeneous mixture that differences in turnover rates and amino acid composition would cancel out. Fractionation of such protein mixtures with ammonium sulphate, which was previously carried out (Campbell, 1955) on chick-liver slices after incubation with [^{14}C]glucose, showed no very striking differences in the radioactivity of the various fractions; nevertheless this possibility requires further investigation.

If proteins are synthesized from free amino acids and if the size of the pools of intermediates containing amino acids is very small, then it would be

expected that all the constituent amino acids would be incorporated into any given protein at the same rate. If, for the purpose of discussion, it is assumed that this is so and that there is no amino acid/protein-bound amino acid exchange, then the free amino acid/protein-bound amino acid ratio (F/P) would be expected to be the same for any one tissue no matter which amino acid was injected. The fact that such ratios for alanine and glutamic acid (Table 3) are in every case greater than those for lysine is a reflexion of the fact that glucose and not amino acid was injected. A delay in the time–activity curve for glutamic acid and alanine derived from glucose compared with that for lysine would, in any case, be expected.

An attempt has been made to express the rate of formation of alanine and glutamic acid from glucose by dividing the ratio of the free lysine/protein-bound lysine by the corresponding ratio for alanine and glutamic acid. The results are shown in Table 3. From these results we may conclude that the conversion of glucose into alanine occurs at approximately the same rate in liver and liver tumour, but is rather slower in kidney. Similarly, the rate of conversion of glucose into glutamic acid is rather lower in liver tumour than in liver, and is very much lower in kidney compared with liver. The results also show that the rate of formation of alanine from glucose in the three tissues is greater than the rate of formation of glutamic acid.

It may be concluded that if in fact the carbohydrate metabolism of liver tumour differs from liver *in vivo* these differences are not reflected as differences in the rate of conversion of glucose into amino acids in the two tissues. It should be emphasized that the liver tissue used in these experiments was taken from livers which were infiltrated with tumours. This tissue, which might be better described as precancerous, may differ in its metabolism from normal liver.

SUMMARY

1. Rats bearing liver tumours that had been induced by feeding *p*-dimethylaminoazobenzene were injected intravenously with a solution containing [^{14}C]glucose and [^{14}C]lysine. One hour after injection the animals were killed and soluble-protein fractions prepared from homogenates of the liver, liver tumour and kidney.

2. The soluble-protein fractions from the tissues were treated with trichloroacetic acid. The protein precipitate was hydrolysed and lysine, glutamic acid and alanine were isolated. The radioactivity of these amino acids was determined.

3. Free lysine, glutamic acid and alanine present in the supernatants from the trichloroacetic acid precipitates were isolated and their radioactivity was determined.

4. It is suggested that after the injection of a radioactive amino acid the ratio of the radioactivity of the free amino acid to that of the protein-bound amino acid in the tissue is a measure of the rate of synthesis of the proteins in the tissue. On this basis the rate of protein synthesis in the three tissues studied was, in descending order: kidney, liver, liver tumour.

5. From a comparison of the ratio of the radioactivity of the free amino acid to that of the protein-bound amino acid for glutamic acid and alanine compared with that of lysine it is concluded that (*a*) the rate of formation of alanine from glucose is similar for liver and liver tumour, but a little lower for kidney; (*b*) the rate of formation of glutamic acid from glucose in liver tumour is a little lower than that for liver, whereas for kidney it is much lower than for liver; (*c*) the rate of formation of alanine from glucose in the three tissues is greater than the rate of formation of glutamic acid from glucose.

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Studies on the Sea Anemone *Gyrostoma* sp.

LIPIDS OF *GYROSTOMA* SP.

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Very little is known about the lipid content of the sea anemone, particularly of those found in tropical waters. This work was undertaken with a view to determining the concentration of lipids and allied substances in the sea anemone, *Gyrostoma* sp., found along the shores of Bombay.

EXPERIMENTAL

The sea anemones were collected during low tide on the beach near the Institute and were preserved in a live condition in aquaria till used for extraction.

Extraction and analysis

The sea anemones were dissected and as much water as possible was removed by drying them between filter papers. Some anemones were used whole for extraction, and in the others the gonads, mesenteries and tentacles were removed and extracted separately. In all these determinations, a small part of the anemone was always dried in an oven at 110° to determine the moisture content.

Either the whole anemone (20–25 g.) or the different organs (5–10 g.) were extracted with ethanol and ether by the method described by Johnson, McNabb & Rossiter (1948), with the difference that the quantities of ethanol and ether for each extraction were adjusted to make the final volume up to 4 l. The same procedure of Johnson *et al.* (1948) was followed for removing the solvent and precipitating the phosphatides in three separate 400 ml. samples of the ether–ethanol extract. The phospholipids were made up to 25 ml. with methanol–ether (1 : 1, by vol.) and the acetone washings up to 50 ml.

All analyses were done in triplicate.

Free and total cholesterol. These were estimated in 2 ml. of the acetone solution by the modified method of Schoenheimer & Sperry (1934), incorporating the improvements suggested by Sperry & Brand (1943) and Sobel & Mayer (1945). The colour was read in a Beckman spectrophotometer (DU Model) at 620 mμ.

Cerebrosides. The method of Brand & Sperry (1941) was used, the galactose liberated on hydrolysis being estimated by the procedure of Miller & Van Slyke (1936). Six 50 ml. samples were taken from the original ether–ethanol solution and, of these, three were hydrolysed and the others treated in exactly the similar way except that hydrolysis was omitted. The quantity of cerebrosides was calculated from the following equation:

$$\text{Wt. of cerebrosides (mg.)} = 4{\cdot}55 \times 0{\cdot}147(A - B) - (C - D)$$

in which A, B, C and D are the volumes of 3 mM ceric sulphate solution required to titrate the hydrolysed lipid sample, hydrolysed blank, unhydrolysed lipid sample and unhydrolysed blank respectively. The factor 4·55 was used on the assumption that approximately equal quantities of phrenosin and kerasin were present. The factor for phrenosin is 4·6 and for kerasin it is 4·5.

Phospholipids. These were estimated in the methanol–ether solution by the method of Hack (1947), in which it has been observed that the mono-aminophospholipids lecithin and kephalin are completely hydrolysed by N-KOH in 18 hr. at 37° and that no hydrolysis of sphingomyelin takes place under these conditions. The total acid-soluble P is a measure of the lecithin and kephalin present (Schmidt, Benotti, Hershman & Thannhauser, 1946) and choline a measure of the lecithin (Hack, 1947). The difference between the total P and the total acid-soluble P in the hydrolysate is a measure of sphingomyelin, and the difference between the molecular equivalents of mono-aminophospholipid and choline gives the molecular equivalent of kephalin.

For the estimation of total P, 1 ml. was taken from the 25 ml. volumetric flask containing the methanol–ether solution of the phospholipids and the total P determined by the method of Sterges, Hardin & MacIntire (1950). The phospholipid was calculated on the assumption that it contained 4·0% of phosphorus.

The remaining 24 ml. of the methanol–ether solution was evaporated under reduced pressure at 60° to a volume of 0·2 ml. After the addition of 5 ml. of N-KOH the tube was stoppered and shaken at 37° for 18 hr. To 0·5 ml. of this solution, pipetted into a centrifuge tube, 0·1 ml. of 5 N-HCl and 3 ml. of 10% (w/v) trichloroacetic acid were added. The centrifuge tubes were stoppered, shaken and allowed to stand at room temperature for 1–2 hr. After centrifuging, the solution was filtered through a Whatman no. 44 filter paper and the total P (mono-aminophospholipid P) estimated in 2 ml. of the trichloroacetic acid filtrate (representing 1·28 ml. of the methanol–ether solution) by the method of Sterges *et al.* (1950). The mono-aminophospholipid was calculated on the assumption that it contained 4·0% of phosphorus.

Lecithin. An estimate of the lecithin present was obtained by determining choline in the remainder of the hydrolysate according to the method of Glick (1944). The colour density was measured in the Beckman spectrophotometer at 526 mμ. Lecithin was calculated as choline (as choline chloride) × 5·56 in the filtrate.

Kephalin. This was taken as representing all the non-choline-containing phospholipids and was calculated as the difference between the mono-aminophospholipid and lecithin.

Sphingomyelin. This is a diaminophospholipid and was calculated as the difference between the total phospholipid and the mono-aminophospholipid.

Table 1. *Lipid composition of the whole anemone, gonads, mesenteries and tentacles of the sea anemone,* Gyrostoma *sp.*

Each value is the mean of three determinations made on each of four batches of material; standard deviations are also given.

	Cerebroside		Cholesterol: Free		Cholesterol: Ester	
	% of dry wt.	% of total	% of dry wt.	% of total	% of dry wt.	% of total
Whole anemone	0·83±0·03	14·78±0·77	0·23±0·02	4·07±0·40	0·25±0·03	4·37±0·45
Gonads	0·59±0·03	6·79±0·45	0·80±0·06	9·18±0·48	0·43±0·04	4·89±0·33
Mesenteries	0·89±0·03	16·04±0·30	0·23±0·05	4·10±0·78	0·29±0·08	5·25±1·29
Tentacles	0·12±0·02	6·38±0·38	0·09±0·01	4·60±0·44	0·05±0·01	2·72±0·49

	Phospholipid: Lecithin		Phospholipid: Sphingomyelin		Phospholipid: Kephalin	
	% of dry wt.	% of total	% of dry wt.	% of total	% of dry wt.	% of total
Whole anemone	3·08±0·11	55·08±1·33	0·25±0·01	4·42±0·19	0·97±0·10	17·30±1·65
Gonads	4·86±0·13	55·62±1·54	0·18±0·02	2·12±0·25	1·87±0·13	21·40±1·24
Mesenteries	2·83±0·09	50·68±1·44	0·39±0·04	7·10±0·90	0·94±0·07	16·86±0·85
Tentacles	1·12±0·14	60·20±3·87	0·12±0·02	6·46±0·40	0·37±0·11	19·64±4·16

RESULTS

The mean concentration of cerebrosides, cholesterol, total phospholipids, the individual phospholipids lecithin, kephalin and spingomyelin and the lipid fractions mentioned above expressed as a percentage of the essential lipid, i.e. as a percentage of the sum of the total cholesterol, cerebrosides and total phospholipids, as well as the standard deviation, are presented in Table 1. The results expressed are the means of the readings obtained with four different batches of material. Each of the four analyses was carried out in triplicate.

From Table 1 it can be seen that the gonads contain most phospholipid, the tentacles containing the least. The mesenteries contain only slightly less phospholipid than the gonads. Cerebrosides are found most in the mesenteries and least in the tentacles. Cholesterol, both combined as well as free, is contained more in the gonads than in any other organ.

The concentration of lecithin and kephalin is greater in the gonads than in any other organ. However, the sphingomyelin content of the gonads is very low, the mesenteries having the greatest concentration. The tentacles contain the least amount of phospholipids.

When expressed as a percentage of the essential lipid, i.e. as a percentage of the sum of the total cholesterol, cerebrosides and total phospholipid, it is seen that the tentacles contain more phospholipid as lipid than any other organ. The gonads contain relatively more cholesterol and cerebrosides. Of the individual phospholipids, lecithin forms the maximum percentage in the tentacles. The sphingomyelin content on this basis is greatest in the mesenteries and least in the gonads.

DISCUSSION

There are almost no reports in the literature on the distribution of phospholipids in the different organs of the sea anemone.

Cholesterol has been shown to be a precursor of progesterone, one of the female sex hormones (Bloch, 1945). The presence of such high concentrations of cholesterol in the gonads, the reproductive organs of the sea anemone, is therefore significant.

The concentration of certain lipid constituents, e.g. sphingomyelin and cerebrosides, may indicate how much of the lipid present in the tissue is of neural origin (Kaucher, Galbraith, Button & Williams, 1943). There is a possibility that the mesenteries, which contain the maximum concentration of these two constituents, have much of their lipid of neural origin.

An important function of the phospholipids is to carry choline as well as fatty acids. In the higher animals choline plays an important part in the mobilization of fat from the liver. The gonads seem to be the main depots of fat in the sea anemone. Choline may be useful in the carrying of fat from the gonads to the other organs. The presence of such high concentration of choline (lecithin) in the mesenteries is significant in that it may be helpful in the digestion of the fats.

SUMMARY

1. The concentrations of cerebrosides, free and total cholesterol, total phospholipid, lecithin and sphingomyelin have been determined in the whole anemone, *Gyrostoma* sp., as well as in its mesenteries, gonads and tentacles.

2. A great variation in lipid content of the various organs of the sea anemone was found, according to the physiological functions they perform, the gonads containing the maximum amount of essential lipid.

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Studies of Sebum

7. THE COMPOSITION OF THE SEBUM OF SOME COMMON RODENTS*

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In order to study the mechanism by which the various components of sebum are formed a suitable laboratory animal was required. Ideally such an animal should have a sebum whose composition closely resembles that of man, but, with the exception of the sheep, little is known about the composition of the hair lipids or sebum of animals. A study was therefore made of the composition of the sebum of the common laboratory animals, namely the guinea pig, mouse, rabbit and rat. The purpose of the investigation was to find how widely the composition of the sebum differed from that of man, so that their uses and limitations as experimental animals could be assessed. A preliminary report of part of this work has already been published (Wheatley, 1953).

EXPERIMENTAL

Materials

In order to obtain a material which would compare with the sebum collected from human subjects the following method of collection was adopted. The mouth and anal regions of the freshly killed animals were cleaned with chloroform, and the body was suspended horizontally over a glass tank. A quantity of acetone (previously twice distilled in all-glass apparatus) was poured over the back of the animal in such a manner that as little as possible flowed over the ears, mouth and anal regions. The acetone which collected in the glass tank was poured back over the animal several times and the animal finally washed with a fresh quantity of acetone. With mice a slightly different procedure was adopted, owing to their smaller size and the small amount of sebum collected from each animal. These animals were simply held by the tail, after the mouth and anal regions had been cleaned with chloroform, and were immersed in a beaker containing acetone. The acetone extract from a number of animals was evaporated to dryness, and the residue dissolved in chloroform. This solution was filtered to remove cellular and other debris and then evaporated to dryness. The amount of material obtained by this procedure from a single animal was approximately as follows: guinea pig 250 mg., mouse 10 mg., rabbit 250 mg., rat 100 mg.

Analytical methods

Chemical constants. Iodine values, acid values, phosphorus and nitrogen were determined by the methods referred to in earlier work (Wheatley, 1954). Cholesterol and 'fast-acting' sterols were determined by the method described by Moore & Baumann (1952). The acrolein method (MacKenna, Wheatley & Wormall, 1950) was used to test for the presence of glycerol.

Fractionation. The fractionation into free fatty acids, combined fatty acids and unsaponifiable matter followed the procedure already described for human sebum (MacKenna *et al.* 1950).

Gas–liquid chromatography. The mixed fatty acids were esterified with methanol and sulphuric acid, and the mixed esters applied directly to the gas chromatogram with a micro-pipette. The general procedure adopted has been

* Part 6: James & Wheatley (1956).

described previously (James & Martin, 1956; James & Wheatley, 1956), unsaturated acids being removed by bromination. The column stationary phase was a high-boiling lubricating-oil extract and the temperature was maintained constant at 197° with boiling ethylene glycol. Peak areas were measured by triangulation and were corrected as described by James & Wheatley (1956).

Chromatography of the unsaponifiable matter. The unsaponifiable matter was fractionated on columns of alumina as described for human sebum (MacKenna, Wheatley & Wormall, 1952). In most cases a sample of 1 g. was fractionated on a 30 g. column of alumina (Brockmann Grade III). Blocking of the column during the initial loading proved particularly troublesome, especially with the mouse-sebum unsaponifiable matter, and was partly prevented by covering the top of the column with a thin layer of sand.

RESULTS

General composition

The general composition and properties of the sebum of these animals are shown in Table 1. It is noteworthy that in all cases only traces of glycerol were obtained on saponification; triglycerides are therefore present only in minute amounts, probably as contaminants. There are, however, marked differences in their properties and in the relative proportions of the different groups of constituents present in the individual seba.

Fatty acids

In Fig. 1 is shown the result obtained by gas–liquid chromatography of 4·8 mg. of the methyl esters of the acids present in rabbit sebum. The chromatogram is most striking in showing the presence of (*a*) large amounts of the straight-chain odd-numbered acids: peak 5, *n*-undecanoic acid; peak 8, *n*-tridecanoic acid; peak 12, *n*-pentadecanoic acid; peak 18, *n*-heptadecanoic acid; (*b*) large amounts of acids that from their position would be expected to possess a highly branched chain: peak 13, a highly branched C_{16} acid; peak 16, a highly branched C_{17} acid; peak 19, a highly branched C_{18} acid. The rabbit apparently produces a highly saturated sebum, since on bromination of the mixture the only peak to disappear is 20, which from its position can be assumed to be oleic acid.

Fig. 2 shows the result obtained with the sample of fatty acids from the rat. The chromatogram is quite distinct from that shown in Fig. 1. In particular the odd-number straight-chain fatty acids are much smaller in amount, the highly branched acids of chain length C_{16}, C_{17} and C_{18} are absent, more unsaturated C_{18} acids are present and much larger amounts of even-numbered *iso*- or *anteiso*-acids are present. Rabbit sebum appears to contain no acids of chain length greater than C_{18}, as the whole of the sample placed on the column can be accounted for in terms of the acids shown in Fig. 1. With rat sebum, however, appreciable amounts of highly branched and simply branched C_{19} and C_{20} acids are present (see Table 2), and acids of chain length up to C_{20} account for only 90% of the material applied to the column.

Mouse sebum (Table 2) contains still larger amounts of acids of chain length greater than C_{21}, only 62% being accounted for in the range C_{16}–C_{21}. This animal is noteworthy in producing larger amounts of an unsaturated C_{16} acid than of the corresponding saturated acid. The percentage of unsaturated C_{18} acids present in mouse sebum is, however, lower than that in rat sebum, except for a mono-unsaturated C_{18} acid which is present in comparable amount in the two animals. The presence of 9·2% of *n*-eicosanoic acid and 6·1% of *n*-heneicosanoic acid is an unexpected finding.

The guinea pig, too, produces a highly saturated sebum, of which only 63% can be accounted for in the range C_{16}–C_{20}. Branched-chain acids of chain length C_{15}, C_{17}, C_{19} and C_{20} are prominent. The detailed distribution of the acids is given in Table 2. These results show that the fatty acid composition of the sebum of the four animals is widely different, and taken together with the results published earlier on human sebum (James & Wheatley, 1956) suggest a marked species differentiation of such an order that all five seba could be readily distinguished on the basis of a gas–liquid chromatogram of their component fatty acids.

Table 1. *General composition and properties of the sebum of some common rodents*

	Guinea pig	Mouse	Rabbit	Rat
M.p.	34°	35°	42°	33°
Acid no.	7	14	16	14
Iodine value	33	41	9	29
Cholesterol (total) (%)	19·8	4·5	3·5	5·3
'Fast-acting' sterols (%)	2·0	8·1	0·1	4·1
Lipid P	Nil	Nil	Nil	Nil
Total N (%)	0·31	0·22	0·15	0·45
Fatty acids (free) (%)	6·0	7·5	9·0	7·4
Fatty acids (combined) (%)	49·3	36·7	43·6	51·4
Unsaponifiable matter (%)	44·8	54·6	45·9	41·4
Glycerol	Trace	Trace	Trace	Trace

Fig. 1. Analysis of 4·8 mg. of methyl esters of the acids present in rabbit sebum. Peaks in order of appearance: (1) air introduced in loading column; (2) water present in air in (1) (negative peak); (3) *n*-decanoic acid; (4) branched C_{11} saturated acid; (5) *n*-undecanoic acid; (6) *n*-dodecanoic acid; (7) branched C_{13} saturated acid; (8) *n*-tridecanoic acid; (9) branched C_{14} saturated acid; (10) *n*-tetradecanoic acid; (11) branched C_{15} saturated acid; (12) *n*-pentadecanoic acid; (13) highly branched saturated C_{16} acid; (14) branched C_{16} saturated acid; (15) *n*-hexadecanoic acid; (16) highly branched C_{17} saturated acid; (17) branched C_{18} saturated acid; (18) *n*-heptadecanoic acid; (19) highly branched C_{18} saturated acid; (20) mono-unsaturated C_{18} acid, probably oleic acid; (21) *n*-octadecanoic acid; (22) highly branched C_{19} saturated acid. Column temperature 197°, length 4 ft. Nitrogen pressure 75·5 cm. Hg; nitrogen flow rate 100 ml./min. Stationary phase, aromatic lubricating-oil extract.

Fig. 2. Analysis of 4·8 mg. of methyl esters of the acids present in rat sebum. Peaks in order of appearance: (1) *n*-decanoic acid; (2) branched C_{12} acid; (3) *n*-dodecanoic acid; (4) branched C_{13} acid; (5) *n*-tridecanoic acid; (6) branched C_{14} acid; (7) mono-unsaturated C_{14} acid; (8) *n*-tetradecanoic acid; (9) branched C_{15} acid; (10) *n*-pentadecanoic acid; (11) branched C_{16} acid; (12) mono-unsaturated C_{16} acid; (13) *n*-hexadecanoic acid; (14) branched C_{17} acid; (15) *n*-heptadecanoic acid; (16) highly unsaturated C_{18} acid; (17) branched C_{18} acid; (18) mono-unsaturated C_{18} acid; (19) *n*-octadecanoic acid. Conditions as for Fig. 1.

Table 2. *Quantitative analysis of the acids present in the sebum of some common rodents*

The figures given represent the percentage of each acid present; 'trace' = less than 0·05%.

Acid	Guinea pig	Mouse	Rabbit	Rat
n-Decanoic	Trace	Trace	0·12	Trace
Branched undecanoic	Trace	Trace	Trace	Trace
n-Undecanoic	Trace	Trace	0·3	Trace
Branched dodecanoic	Trace	Trace	Trace	Trace
n-Dodecanoic	Trace	Trace	2·2	Trace
Branched tridecanoic	Trace	Trace	0·6	Trace
n-Tridecanoic	Trace	Trace	6·6	Trace
Branched tetradecanoic	Trace	0·2	0·7	2·6
Mono-unsaturated C_{14}	Trace	0·4	0	0·5
n-Tetradecanoic	0·3	0·7	10·7	1·6
Branched pentadecanoic	0·7	0·3	1·3	1·1
Mono-unsaturated C_{15}	0	0·7	0	0
n-Pentadecanoic	1·6	1·1	6·0	0·8
Highly branched hexadecanoic	0	0	1·8	0
Branched hexadecanoic	0·5	1·7	0·5	4·7
Mono-unsaturated C_{16}	1·1	7·3	0	2·1
n-Hexadecanoic	15·8	5·0	24·0	17·4
Highly branched heptadecanoic	0	0	11·0	0
Branched heptadecanoic	2·4	0·9	1·7	5·0
Mono-unsaturated C_{17}	0·8	0·9	0	0
n-Heptadecanoic	5·8	1·7	6·8	3·1
Highly branched octadecanoic	3·1	0	14·3	0
Branched octadecanoic	Trace	0·7	0	11·3
Highly unsaturated C_{18}	0	0	0	1·7
Di-unsaturated C_{18}	0	0	0	3·1
Mono-unsaturated C_{18}	7·7	10·0	3·7	11·3
n-Octadecanoic	4·0	3·9	8·2	8·9
Branched nonadecanoic	9·8	1·0	0	3·1
Mono-unsaturated C_{19}	3·1	0	0	0
n-Nonadecanoic	2·2	2·2	0	0
Highly branched eicosanoic	0	0	0	4·1
Branched eicosanoic	4·3	5·1	0	6·8
n-Eicosanoic	Not measured	9·2	0	Not measured
Branched heneicosanoic	Not measured	2·5	0	Not measured
n-Heneicosanoic	Not measured	6·1	0	Not measured

Table 3. *Chromatography of the unsaponifiable matter of the sebum of common rodents*

Fraction*	Guinea pig (%)	Mouse (%)	Rabbit (%)	Rat (%)
I	3·4	2·1	7·5	3·6
II	12·8	10·2	74·5	42·8
III	42·3	23·6		24·3
IV	37·9	51·4	12·9	21·4
V	3·6	2·0	2·6	3·8
	—	10·7†	2·2†	3·1†

* See text. † Not eluted from column.

Unsaponifiable matter

The unsaponifiable matter was fractionated on columns of alumina into the five principal fractions indicated in Table 3, which were eluted with light petroleum (b.p. 40–60°), 5% and 10% chloroform (v/v) in light petroleum, chloroform and methanol in this order. These fractions were then examined in greater detail with the following results.

Fraction I. The materials from all four animals were soft white waxes consisting entirely of hydrocarbons. They had a relatively low iodine value (19, 20, 7 and 36 for the material from the guinea pig, mouse, rabbit and rat respectively) and it was not possible to demonstrate the presence of squalene by isolation of the hexahydrochloride. This does not exclude the possible presence of small amounts, and if all the unsaturated material present were squalene it would constitute 5·1, 5·4, 1·8 and 9·7% of the fractions from the guinea pig, mouse, rabbit and rat respectively. This would amount to only 0·17, 0·11, 0·13 and 0·35% respectively of the total unsaponifiable matter of the sebum from these animals.

Fractions II and III. Owing to the presence of the 'fast-acting' sterols a clear-cut separation of the sterols and wax alcohols was not achieved. It was found more convenient to pool both fractions and then remove the sterols by means of digitonin. The sterols were recovered from the precipitated digitonides by refluxing with pyridine for 2 hr., followed by treatment with ether in the usual manner. The wax alcohols were recovered from the mother liquors and washings from the digitonide precipitation after removal of the excess of digitonin. They constituted 26·1, 31·9, 92·1 and 63·3%

of the pooled fractions from the guinea pig, mouse, rabbit and rat respectively. Urea-adduct formation (Tiedt & Truter, 1952) showed the presence of 1·6, 1·4, 66·0 and 25·6 % of straight-chain alcohols respectively in the alcohols isolated from these animals, and both saturated and unsaturated alcohols were present in all cases. An attempt to examine these alcohols in greater detail by gas–liquid chromatography was unsuccessful, since the materials appeared to have a chain length greater than C_{18} and were beyond the range of the instruments at present available. They will be further examined at a later date. Recrystallization of the alcohols from the rabbit gave a white wax, m.p. 75·6° (Found: C, 80·8; H, 13·7 %), indicating an average chain length of about C_{24}. Even this brief examination has, however, indicated that the alcohols from the four animals are different.

The sterols isolated from the guinea pig, mouse, rabbit and rat contained respectively 9·2, 63·9, 2·8 and 43·6 % of 'fast-acting' material when examined by the method of Moore & Baumann (1952). This 'fast-acting' material has not been isolated, but, as a result of the identification by Idler & Baumann (1952) of the principal 'fast-acting' sterol in rat skin as cholest-7-en-3β-ol (lathosterol), they have been provisionally identified as this sterol. Examination of the ultraviolet spectra of these fractions showed the presence of 0·06 % of 7-dehydrocholesterol in the fraction from the mouse. The fraction from the rat gave an indication of a faint trace (less than 0·01 %), but those from the guinea pig and rabbit failed to show detectable amounts of 7-dehydrocholesterol.

Fraction IV. Recrystallization of the fraction from the mouse gave white feathery needles, m.p. 85°. Periodate oxidation indicated that this was a glycol, and lead tetra-acetate oxidation (Horn & Hougen, 1953) yielded formaldehyde, characterized as the dimedone complex (m.p. and mixed m.p. 189°), and long-chain fatty acids which were not further studied. The fraction from the mouse therefore consisted almost entirely of either a single alkane-1:2-diol or a mixture of several closely related diols. The average chain length appeared to be about C_{16}. (Found: C, 74·7; H, 13·5; $C_{16}H_{34}O_2$ requires C, 74·4; H, 13·2 %.) No sterols were present in either the crude fraction or the material obtained from the mother liquors after removal of most of the diol, as indicated by the Liebermann–Burchardt test.

The corresponding fractions from the guinea pig, rabbit and rat, when analysed by the periodate method of Karnovsky & Rapson (1946), were found to contain 66·4, 38·2 and 33·4 % respectively of diols (calculated as the C_{20} diol). Each fraction on oxidation with lead tetra-acetate yielded formaldehyde (dimedone complex), together with long-chain fatty acids, indicating that all contained alkane-1:2-diols.

In addition to these diols the fractions from the guinea pig and the rabbit contained sterols, indicated by the Liebermann–Burchardt test, which constituted 3·9 and 5 % respectively of the crude fractions and were 'fast-acting'. Both materials gave positive Rosenheim and antimony trichloride tests. In both cases the colour in the Rosenheim test was an intense royal blue; with the antimony trichloride test the guinea-pig material gave a purple coloration, but the rabbit material a blue. Paper chromatography (alumina-impregnated paper, chloroform as solvent; Wheatley, 1954) indicated two components in both materials, both giving blue spots with antimony trichloride. Neither fraction gave a digitonide, nor was there any distinctive band in the ultraviolet spectrum.

The fraction from the rat gave a reddish-brown coloration with the Liebermann–Burchardt test. The absorption spectrum of the coloured material showed a broad band at about 350 mμ. The corresponding spectrum for the fraction from the guinea

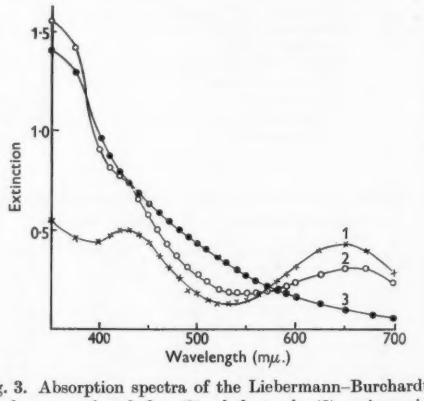

Fig. 3. Absorption spectra of the Liebermann–Burchardt colours produced by (1) cholesterol, (2) guinea-pig fraction IV, (3) rat fraction IV.

Table 4. *Estimated composition of the unsaponifiable matter of the sebum of common rodents*

	Guinea pig (%)	Mouse (%)	Rabbit (%)	Rat (%)
Hydrocarbons	3·4	2·1	7·5	3·6
Wax alcohols	11·1	10·8	68·7	42·5
Alkane-1:2-diols	25·1	50·4	4·9	7·1
Cholesterol	40·0	8·3	7·6	13·9
'Lathosterol'	4·0	14·8	0·2	10·7
Other sterols	1·5	—	0·6	0·4
Unidentified	14·9	13·7	10·5	21·8

pig showed a similar band in addition to the usual band at 560 mμ. (Fig. 3), but no indication has yet been obtained of the nature of the substance responsible for this coloration.

Fraction V. These were dirty-white or brown waxes or gums. The fractions from the guinea pig, mouse and rabbit contained little or no sterol material. That from the rat gave a strongly positive Liebermann–Burchardt test, a positive Rosenheim test but a negative antimony trichloride test. This material did not yield a digitonide, nor did it show distinctive ultraviolet absorption bands; it comprised about 5% of the crude fraction.

From the results of this detailed examination of the various fractions the composition of the unsaponifiable matter of the sebum of these animals can be calculated (Table 4).

DISCUSSION

An examination of the sebum of four common types of rodents used as laboratory animals has revealed some striking differences in composition. In general the materials differ from human sebum in that they contain little or no squalene, and differ from the sebum of the sheep and other ruminants in that they contain little or no 'isocholesterol'. Instead these rodents' sebum appears to contain lathosterol. The amount present in rabbit sebum is rather small (0·1%). This sterol gives a much more intense coloration in the Liebermann–Burchardt test than does cholesterol; consequently earlier workers have obtained erroneously high results for the cholesterol content of the hair lipids from certain animals (Eckstein, 1927).

Three classes of long-chain aliphatic compounds, namely hydrocarbons, alcohols and fatty acids, have already been shown to be present in sebum. To these may now be added a further class, the alkane-1:2-diols. These have already been shown to be present in sheep wool-wax (Horn & Hougen, 1953) and are now shown to be present in the sebum of the four animals examined here, accounting for between 5 (rabbit) and 50% (mouse) of the unsaponifiable matter. In the sheep, Horn & Hougen identified five such diols, namely *n*-hexadecane-, 16-methylheptadecane-, 18-methylnonadecane-, 20-methylheneicosane- and 22-methyltricosane-1:2-diol. In the rodents here examined it is also possible that similar homologous mixtures exist and it is intended to make a further study of the precise nature of the diols present in each animal sebum.

Examination of the fatty acids by gas–liquid chromatography has shown the nature of these acids to be characteristic for each of the animals. In many ways the acids are unusual; for instance, 27% of the acids from rabbit sebum are highly branched acids. Preliminary examination also indicates that the alcohols from each animal are different. It is also likely that both the hydrocarbons and the diols will be shown to be different for each animal.

In addition to differences in the carbon chains of these long-chain compounds there are differences in the proportions of the types of compounds present in the unsaponifiable matter. Thus the unsaponifiable matter from rabbit and rat sebum contains a high proportion of alcohols (68·7 and 42·5% respectively); that from the mouse contains large amounts of diols (50·4%), and that from the guinea pig a high proportion of cholesterol (40·0%).

In general the composition of sebum seems to be a unique property of the species from which it comes. The marked differences in the composition of the sebum from closely related animals makes the selection of experimental animals difficult, nevertheless these differences can be turned to advantage in some cases. For instance, for studies on the waxes of sebum the rabbit would be the animal of choice, while the mouse should be used for studies of the diols. The common laboratory animals can be used for a large number of metabolic studies on the formation and secretion of sebum. There are, however, many purposes for which they cannot be used, e.g. for studies on squalene, and the examination of the composition of the sebum from a number of other species will be necessary if such problems are to be studied.

SUMMARY

1. The sebum of the common rodents, the guinea pig, mouse, rabbit and rat, has been examined and the usefulness of these animals for experimental studies on sebum metabolism assessed.

2. Marked differences in the composition of the sebum of these four animals have been demonstrated, both among themselves and from that of man and the sheep. The presence of lathosterol (cholest-7-en-3β-ol), however, appears to be characteristic of the sebum of the species of this family.

3. A fourth group of long-chain aliphatic compounds, namely alkane-1:2-diols, has been established as a common constituent of sebum.

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The Separation of the C_6–C_{12} Fatty Acids by Reversed-phase Partition Chromatography

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The monoterpene (C_{10}) acids are becoming of increasing interest to biochemists as possible intermediates in the biosynthesis of cholesterol, rubber and the carotenoids. The isolation and separation of small quantities of terpene acids should be facilitated by chromatography. Since these acids are all somewhat more polar than *n*-decanoic acid a chromatographic system capable of separating the straight-chain C_6–C_{10} acids should be appropriate for their resolution.

Partition chromatography has been successfully applied to the separation of the C_5–C_{10} fatty acids (Ramsey & Patterson, 1948; Peterson & Johnson, 1948; Boldingh, 1950; Nijkamp, 1951; Vandenheuvel & Hayes, 1952; Zbinovsky, 1955), and to the acids in the lower part of this range, C_6–C_8 (Moyle, Baldwin & Scarisbrick, 1948; Fairbairn & Harpur, 1951; Popják & Tietz, 1954). The lower fatty acids can also be separated by displacement analysis (Holman & Hagdahl, 1950). Gas–liquid chromatography affords an analytical separation with the highest resolution (James & Martin, 1952, 1956). Nevertheless, the convenience, high resolving power and larger capacity of reversed-phase partition chromatography as developed by Howard & Martin (1950) justified extending this method to include the C_6–C_{12} acids.

Crombie, Comber & Boatman (1955) decreased the acetone concentration in the aqueous acetone–medicinal paraffin solvent system devised by Howard & Martin, and thereby extended the separations to include decanoic and octanoic acids. However, the aqueous methanol–chloroform system tentatively tested by Howard & Martin (1950) and developed by Wittenberg (1955) for another purpose was found particularly well suited to the separation of the C_6–C_{12} acids.

Columns of siliconized kieselguhr prepared with aqueous methanol–chloroform tend to lose the stationary phase during use (Howard & Martin, 1950). This difficulty may be obviated by the addition of a hydrocarbon to the chloroform phase (Bergström & Sjövall, 1951). Presumably the hydrocarbon achieves its effect by increasing the interfacial tension between the solvent phases (Howard & Martin, 1950). The stability of the column improves with increasing molecular weight of the added hydrocarbon. 2:2:4-Trimethylpentane stabilizes the columns far more than hexane or heptane, and decane affords a column which is stable to repeated use. Accordingly, a decane fraction, Skellysolve S, has been used to stabilize the columns described.

METHODS

Preparation of kieselguhr. Hyflo Super Cel (Johns-Manville Co. Ltd., New York, U.S.A.) was washed by decantation, twice with 3 vol. of 0·1 N-HCl (Sjövall, 1953) and repeatedly with water until the supernatant fluid was relatively free of fine particles, which otherwise would leak from the columns (Silk & Hahn, 1954). The washed kieselguhr was dried at 110°, and, when cool, stirred while passing through it a stream of dry nitrogen previously drawn through liquid dichlorodimethylsilane. The treated kieselguhr was washed with methanol, until the washings were no longer acid, was dried at 110° and stored for use.

Solvents. Solvents were used without special purification. Skellysolve S (boiling range 150–202°) is stated by the manufacturer (Skelly Oil Co., Kansas City, Missouri, U.S.A.) to contain some nonanes and undecanes and a considerable quantity of *n*- and *iso*-decanes.

Columns. The columns used were 1·8 cm. in diameter. Treated kieselguhr (15 g.) and the less polar solvent phase (13 ml.) were stirred together until a homogeneous powder was obtained. This was suspended in the more polar phase and packed into the column with the aid of a perforated stainless-steel plunger as described by Howard & Martin (1950). The column obtained was 11 cm. long. The solvent was run down the column at the rate of 30 ml./hr., and collected in 3 ml. fractions. Columns may be used repeatedly but must be protected from fluctuations in room temperature which would disturb the mutual solubilities of the solvent phases.

Loading. The acids to be resolved were added to the column in solution in the more polar solvent phase containing not more than 15 mg. of acids/ml. A total of about 0·2 m-mole of acids may be separated, although the capacity for dodecanoic acid is somewhat more limited. The quantity of acids which may be separated is limited by solvent–solute interactions, which cause an increase in the relative volume of the less polar phase.

Titration. The effluent fractions were titrated with methanolic 0·1 N-NaOH, delivered from a microburette, to the end-point of phenol red.

Solvent system. Of the several tried, only two solvent systems were found useful. The mixture chloroform–Skellysolve S–water–methanol (8:1·5:12:20, by vol.), served to separate acids up to C_{10}, as exemplified in Figs. 1 and 3. The mixture of the same components in the proportion 8:1·75:11:20 (by vol.) served to extend the separation to include dodecanoic acid (Fig. 2). Either solvent mixture may be used to separate geranic acid from other acids (Fig. 3). The solvents were prepared by mixing the four components, allowing the mixture to cool, and separately filtering the two phases through dry filter paper before use. The less polar, chloroform-rich, lower phase served as the stationary phase; the more polar, aqueous methanol, upper phase served as the moving phase of the column.

RESULTS AND DISCUSSION

The separation of *n*-hexanoic, *n*-octanoic, *n*-decanoic and dodecanoic acids is illustrated in Figs. 1 and 2. *n*-Valeric and *n*-hexanoic acids are partially resolved; *n*-butyric acid moves close to the solvent front. The recoveries are about 90 %.

The terpene acid, geranic acid, is separated from decanoic acid with each of the solvent mixtures described (Fig. 3). The mono- and di-hydroxy terpene acids should travel down the column more rapidly than geranic acid and will be well separated from geranic acid and from each other, since other compounds differing only in the number of their hydroxyl groups are widely separated on chromatograms of this type (Howard & Martin, 1950;

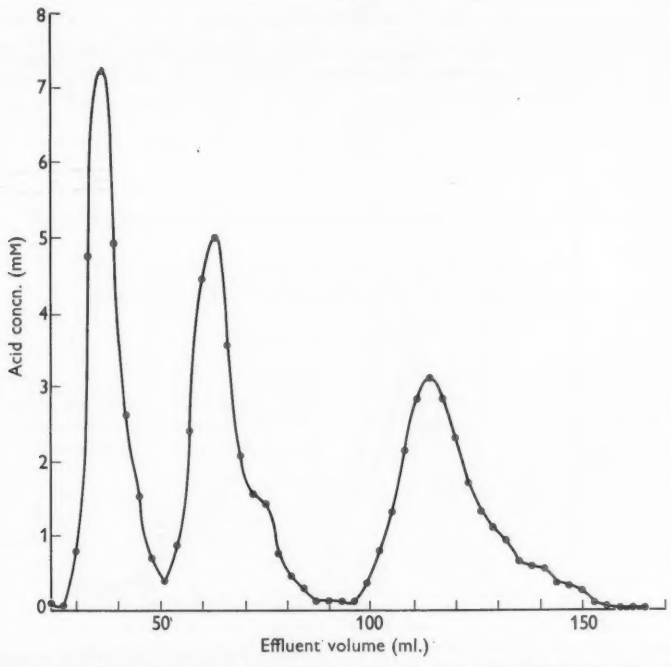

Fig. 1. Separation of a mixture of *n*-hexanoic, *n*-octanoic and *n*-decanoic acids (75 μmoles of each) on a column (1·8 cm. × 12·4 cm.). Solvent system: chloroform–Skellysolve S–water–methanol (8:1·5:12:20, by vol.).

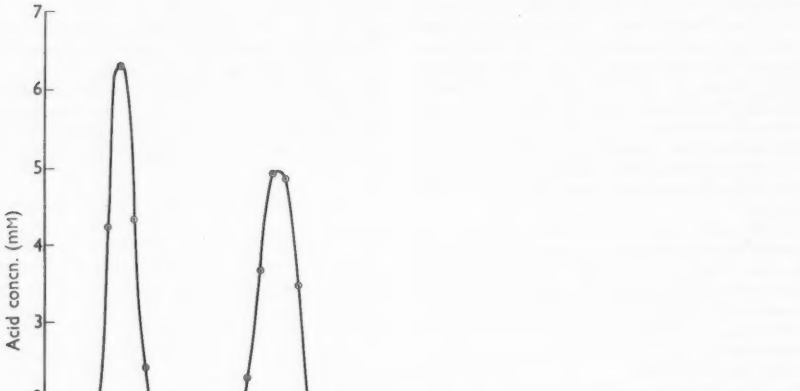

Fig. 2. Separation of a mixture of *n*-octanoic (75 μmoles), *n*-decanoic (75 μmoles) and *n*-dodecanoic acids (61 μmoles) on column (1·8 cm. × 11·7 cm.). Solvent system: chloroform–Skellysolve S–water–methanol (8:1·75:11:20, by vol.).

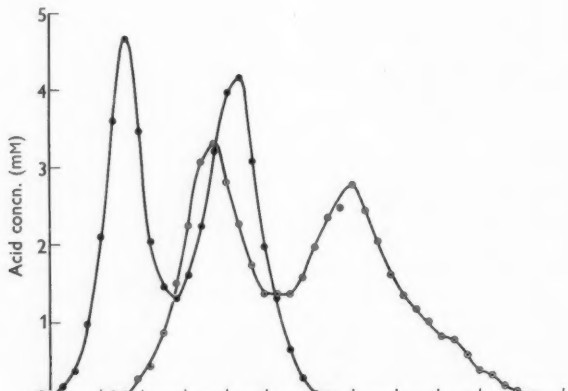

Fig. 3. Separation of mixtures of geranic and *n*-decanoic acids (75 μmoles of each) on columns (1·8 cm. × 12·3 cm.). ⊙, Solvent system as Fig. 1; ●, solvent system as Fig. 2.

Wittenberg, 1955). No terpene acids other than geranic acid were available for testing. However, the position of the peak for any acid may be predicted from an experimentally determined value of the distribution coefficient (α), since the chromatogram described behaves as a theoretical partition chromatogram (Martin & Synge, 1941). The pertinent values necessary for the calculation are: cross-sectional area of column (A), 255 mm.^{2}; cross-sectional area of non-mobile phase (A_s), 85 mm.^{2}; cross-sectional area of mobile phase (A_L), 113 mm.^{2}.

It is of interest that columns similar to those described here have been useful for the separation of three other groups of acidic lipids, the glycerol phosphatides (Garvin & Karnovsky, 1954), the bile acids (Bergström & Sjövall, 1951; Sjövall, 1953), and *N*-succinylsphingosine and its congenors (Wittenberg, 1955).

SUMMARY

1. The method of reversed-phase partition chromatography has been extended to the separation of the C_6–C_{12} fatty acids.

2. This type of chromatography is applicable to the separation of the terpene acids.

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The Synthesis of some Analogues of Glutathione

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Several analogues of glutathione were required for studies on glyoxalase described in a later paper. The preparation of these compounds was undertaken by synthesis from amino acids and by modification of glutathione itself.

King & Kidd (1949, 1951) and King, Jackson & Kidd (1951) have shown that when phthalylglutamic anhydride is condensed with amino acids or peptides γ- and not α-glutamyl derivatives are obtained, so this route was first investigated.

Phthalyl-DL-glutamic anhydride was allowed to react with glycylglycine in acetic acid, giving phthalyl-γ-DL-glutamylglycylglycine. The phthalyl group was then removed with phenylhydrazine (Boissonnas & Schumann, 1952) and, after recrystallization, the resulting γ-DL-glutamylglycylglycine behaved as a pure compound on paper chromatograms. When this compound was heated with ninhydrin, one mole of carbon dioxide was evolved/mole of peptide; since only free carboxyl groups with adjacent free amino groups react in this way, the peptide was evidently γ- not α-glutamylglycylglycine.

In a similar manner, phthalyl-γ-DL-glutamyl-DL-alanylglycine was obtained from DL-alanylglycine, and the removal of the phthalyl group gave γ-DL-glutamyl-DL-alanylglycine. To confirm the identity

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of this peptide, an aqueous solution of glutathione was shaken with Raney nickel, which removed the sulphur. The product was shown, by hydrolysis and paper chromatography, to contain glutamic acid, alanine and glycine. It could not be distinguished chromatographically from the γ-DL-glutamyl-DL-alanylglycine prepared above, although the latter was presumably a mixture of two pairs of diastereoisomers.

The phthalyl intermediates required in these syntheses were tested for purity by paper chromatography in *n*-butanol–pyridine–water (5:2:2, by vol.). Treatment with ninhydrin then revealed any amino acids or peptides. Phthalyl compounds could be detected if the paper were sprayed with 5% (w/v) aqueous hydrazine hydrate, dried thoroughly in an oven at 100° and then examined under ultraviolet light. The phthalylhydrazide so formed showed a characteristic blue fluorescence.

The *S*-methyl derivative of glutathione was obtained by treating the peptide in liquid ammonia with metallic sodium followed by methyl iodide. *S*-Methylcysteine was prepared similarly.

It was already known that *N*-ethylmaleimide reacts with thiol groups (Friedmann, Marrian & Simon-Reuss, 1949; Hanes, Hird & Isherwood, 1950). This reaction was carried out with glutathione and with L-cysteine in aqueous solution and the resulting *S*(*N*-ethylsuccinimido)glutathione and *S*(*N*-ethylsuccinimido)-L-cysteine were isolated.

The sulphydryl group of glutathione was oxidized to a sulphonic acid group by the performic acid reagent of Toennies & Homiller (1942) to give γ-L-glutamyl-L-β-sulphoalanylglycine. This compound, though not crystalline, gave an elementary analysis in reasonable agreement with the theoretical figures.

L-Cysteinylglycine was prepared from glutathione by mild hydrolysis with hydrochloric acid, whereby the labile γ-glutamyl group was removed without fission of the other peptide bond (Olson & Binkley, 1950).

EXPERIMENTAL

All m.p.'s are uncorrected. The hydrolyses of peptides were carried out by heating in a sealed tube with 6N-HCl for 15 hr. at 100°.

Phthalyl-γ-DL-glutamylglycylglycine. Phthalyl-DL-glutamic anhydride (5·9 g.) was dissolved in hot acetic acid (50 ml.) containing glycylglycine (3·0 g.) and the solution set aside for 30 min. The acetic acid was removed under reduced pressure and the syrup dissolved in hot water (10 ml.): the white crystals of phthalyl-γ-DL-glutamylglycylglycine (5 g., 53%), which deposited as the solution cooled, had m.p. 165–167° (decomp.) after drying at 78° *in vacuo*. The product could be recrystallized from water without altering the m.p. but it did not analyse satisfactorily till treated as follows. The crystals were dissolved in ethanol and the solution was dried over anhydrous sodium sulphate. To the hot ethanolic solution, dry ethyl acetate was added till a turbidity developed. Hard masses of crystals of *phthalyl-γ-DL-glutamylglycylglycine*, m.p. 186–188° (decomp.), separated on cooling. (Found: C, 52·2; H, 4·2; N, 10·3. $C_{17}H_{17}O_8N_3$ requires C, 52·2; H, 4·4; N, 10·7%.)

When the anhydrous material was recrystallized from water, the original hydrate, m.p. 165–167°, was obtained. (Found: C, 49·6; H, 4·4; N, 10·4. $C_{17}H_{17}O_8N_3,H_2O$ requires C, 50·0; H, 4·6; N, 10·3%.)

Chromatography of the product on paper in butanol–pyridine–water gave only one spot, R_F 0·25, which reacted with the hydrazine reagent.

γ-DL-Glutamylglycylglycine. Phthalyl-γ-DL-glutamylglycylglycine (3·0 g.) was dissolved in 95% (v/v) ethanol (75 ml.) containing tri-*n*-butylamine (2·8 g.). Phenylhydrazine (1·7 g.) was added, and the solution was refluxed for 3 hr., during which a white precipitate separated. To the warm solution acetic acid (1·4 g.) was added and then ethyl methyl ketone (110 ml.) to complete precipitation. The product was washed with ethyl methyl ketone by decantation and then filtered, giving a white powder (1·1 g., 50%) which had m.p. 180–183° (decomp.). Attempts to purify the compound by precipitation from aqueous solution with ethanol were unsuccessful. However, after a concentrated aqueous solution of the peptide had been passed through a very small column (2·5 cm. × 0·5 cm.) of activated charcoal, the addition of ethanol (10 vol.) to the combined eluate and washings precipitated small white crystals which analysed satisfactorily. (Found: C, 38·3; H, 5·9; N, 15·3. Calc. for $C_9H_{15}O_6N_3,H_2O$: C, 38·7; H, 6·1; N, 15·1%.) (Cf. Le Quesne & Young, 1950.)

The compound was chromatographed in phenol–water (4:1, v/v) in an atmosphere of NH_3 [from a 1% (w/v) solution] and coal gas. On spraying with ninhydrin only one spot appeared, R_F 0·36.

After hydrolysis in 6N-HCl, the products were chromatographed similarly. Two ninhydrin-reacting spots appeared, corresponding to glutamic acid and glycine controls, the glycine spot being about twice as intense as the other.

The compound was analysed for α-amino N by Van Slyke's ninhydrin method. (Found: 4·94. Calc. for $C_9H_{15}O_6N_3,H_2O$: 5·01% α-amino N).

Phthalyl-γ-DL-glutamyl-DL-alanylglycine. Phthalyl-DL-glutamic anhydride (3·6 g.) was dissolved in hot acetic acid containing DL-alanylglycine (2·0 g.) and the solution set aside for 30 min. The acetic acid was removed under reduced pressure, leaving a syrup which was dissolved in hot ethanol. Hot ethyl acetate was added till the solution became cloudy. White crystals of *phthalyl-γ-DL-glutamyl DL-alanylglycine* (3·0 g., 54%) were filtered off next day, m.p. 188–194° (decomp.). Repeated recrystallization from ethanol–ethyl acetate gave a product with m.p. 197–198° (decomp.). (Found: C, 53·2; H, 4·6; N, 10·2. $C_{18}H_{19}O_8N_3$ requires C, 53·3; H, 4·7; N, 10·4%.) Only one spot, R_F 0·30, was obtained when the product was run on paper with butanol–pyridine–water mixture and sprayed with hydrazine hydrate. The compound was very similar to phthalyl-γ-DL-glutamylglycylglycine in its properties except that no crystalline hydrate was obtained.

γ-DL-Glutamyl-DL-alanylglycine. Phthalyl-γ-DL-glutamyl-DL-alanylglycine (1·0 g.) was dissolved in 95% ethanol (30 ml.) containing tri-*n*-butylamine (0·93 g.). Phenylhydrazine (0·54 g.) was added and the solution was refluxed for 3 hr., during which white crystals began to separate.

Acetic acid (4·5 g.) was added, then ethyl methyl ketone (45 ml.), and the solution was left overnight. The precipitate was washed with ethyl methyl ketone by decantation, filtered off and dried in air, giving *γ*-DL-*glutamyl*-DL-*alanylglycine* (0·5 g., 68%), m.p. 195–198°. The compound was purified by the addition of ethanol to its aqueous solution till cloudy. The product, which separated overnight, was dried *in vacuo* at 78° and then had m.p. 198°–200° (decomp.). (Found: C, 41·1; H, 6·4; N, 13·8. $C_{10}H_{17}O_6N_3,H_2O$ requires C, 41·0; H, 6·5; N, 14·3%.) When chromatographed in phenol–water without NH_3 it gave one spot, R_F 0·60; when 1% of NH_3 was present, the R_F fell to 0·50.

When an acid hydrolysate of the compound was chromatographed on paper in phenol–water in the presence of NH_3, three spots were obtained with R_F 0·22, 0·35 and 0·50, respectively, corresponding to glutamic acid, glycine and alanine run simultaneously.

γ-L-*Glutamyl*-L-*alanylglycine from glutathione.* Glutathione (1 g.) was dissolved in water (20 ml.) and Raney nickel C (20 ml. of 0·5 g./ml. aqueous suspension; Hurd & Rudner, 1951) was added. The mixture was shaken for 2 days at 37°, then centrifuged; the supernatant was decanted. The residue was extracted with water and the combined extracts and supernatant were evaporated to dryness *in vacuo*. Only 0·4 g. of a brown residue remained, but no more material could be extracted from the nickel with hot water or with dilute NH_3. A similar loss of product on Raney nickel has been reported by Edward & Martlew (1954).

The brown residue was dissolved in a little water and much of the pigment was removed by precipitation with methanol. The remaining solution still contained some nickel, which was removed by precipitation with H_2S in the presence of NH_3. After the solution had been freed of H_2S and the product purified by precipitation with ethanol, it was examined by paper chromatography in phenol–water with an atmosphere of NH_3. Two ninhydrin-reacting spots were found, a very faint one with R_F 0·11 and a strong one with R_F 0·50, the latter figure being increased to 0·61 when NH_3 was omitted. This behaviour is very similar to that of *γ*-DL-glutamyl-DL-alanylglycine. Confirmation of structure was obtained by hydrolysing the product in 6N-HCl and chromatographing in phenol–water with an atmosphere of NH_3. Three spots were obtained which reacted with ninhydrin and corresponded to glutamic acid, alanine and glycine run simultaneously. A very faint spot also appeared, with R_F 0·10, corresponding to the original impurity.

S-Methylglutathione. Glutathione (0·92 g.) was dissolved in liquid NH_3 (100 ml.) with stirring. To the faintly reddish solution, sodium (approx. 0·21 g.) was added till the resulting indigo colour persisted for at least 1 min. Methyl iodide (0·57 g.) was added dropwise over a period of 5 min. and stirring was continued for a further 15 min. The NH_3 was allowed to evaporate and the resulting solution was dried *in vacuo* over conc. H_2SO_4. The residue was taken up in water, adjusted to pH 3·0 with HI, then mixed with ethanol (3–4 vol.). Next day, amorphous, hygroscopic *S*-methylglutathione (0·67 g., 70%) was filtered off. The product was purified by solution in hot water followed by the addition of an equal vol. of hot ethanol: crystallization occurred after a few hours in the refrigerator. After recrystallization from the minimum of hot water, *S-methylglutathione* was obtained as small white crystals, m.p. 197–199° (decomp.), after drying *in vacuo* at 78°. (Found: C, 41·3; H, 6·1; N, 13·1. $C_{11}H_{19}O_6N_3S$ requires C, 41·1; H, 5·9; N, 13·1%.)

Methylation of glutathione with dimethyl sulphate gave a sticky syrup which could not be crystallized.

The products of each reaction were chromatographically identical, each showing a single spot, R_F 0·53, in phenol–water with an atmosphere of NH_3, and R_F 0·63 in the absence of the NH_3. These spots reacted with ninhydrin and with iodoplatinic acid but not with nitroprusside and cyanide (Block, LeStrange & Zweig, 1952). After hydrolysis in 6N-HCl overnight, each preparation gave three spots on chromatography in phenol–water in the presence of NH_3, corresponding to authentic glutamic acid, *S*-methylcysteine and glycine run simultaneously.

S-Methyl-L-*cysteine.* This compound was prepared by methylation with methyl iodide in liquid NH_3, as for *S*-methylglutathione.

S(*N-Ethylsuccinimido*)-L-*cysteine.* L-Cysteine (0·50 g.) was dissolved in water (10 ml.) with slight warming, and *N*-ethylmaleimide (0·53 g.) was added gradually with shaking. After 1 hr., acetone (3–4 vol.) was added to the solution to precipitate S(*N-ethylsuccinimido*)-L-*cysteine* (0·93 g., 92%), m.p. 185–191° (decomp.). The product, purified by repeated precipitation from aqueous solution, first by acetone then by ethanol, had m.p. 187–193° (decomp.). (Found: C, 44·1; H, 5·7; N, 11·1. $C_9H_{14}O_4N_2S$ requires C, 43·9; H, 5·7; N, 11·4%.) The compound gave only one ninhydrin-reacting spot, R_F 0·87, when run on paper in phenol–water with an atmosphere of NH_3. When the compound was treated with 6N-HCl in a sealed tube overnight, some was unchanged but this was accompanied by a larger amount of a new compound, R_F 0·05, which was presumably a succinic acid derivative of cysteine.

S(*N-Ethylsuccinimido*)*glutathione.* Glutathione (1·0 g.) was dissolved in water (10 ml.) with slight warming and *N*-ethylmaleimide (0·42 g.) was added gradually with shaking. After 1 hr., the product was precipitated with acetone, giving S(*N-ethylsuccinimido*)*glutathione* (1·3 g., 91%), m.p. 198–199° (decomp.). Purification was effected as for *S*(*N*-ethylsuccinimido)cysteine, giving fine white crystals, m.p. 202–203° (decomp.). (Found: C, 44·2; H, 5·4; N, 12·9. $C_{16}H_{24}O_8N_4S$ requires C, 44·5; H, 5·6; N, 13·0%.) When run on paper in phenol–water the compound gave only one spot, R_F 0·75, which reacted with ninhydrin and with the iodoplatinic acid reagent but not with nitroprusside and cyanide. An acid hydrolysate of the compound, run in phenol–water in the presence of NH_3, gave four ninhydrin-reacting spots, R_F values 0·05, 0·25, 0·36 and 0·87. The spots at 0·87 and 0·05 corresponded to *S*(*N*-ethylsuccinimido)cysteine and its breakdown product, referred to above, respectively.

γ-L-*Glutamyl*-L-*β*-*sulphoalanylglycine.* Glutathione (1·0 g.) was dissolved in 98% formic acid (40 ml.) and 30% (w/v) of H_2O_2 (5 ml.) was added. After 1·5 hr. the solution was placed in a vacuum desiccator over KOH and left overnight. The residual syrup was dissolved in dry methanol and a mixture of equal parts of dry ethanol and dry ether was added to give a flocculent precipitate. This could be purified by repeated solution in methanol and precipitation with ethanol–ether to yield an amorphous white powder. When heated, the peptide first shrank and then swelled to a frothy mass as the temperature rose from 120° to 180°, the froth liquefying and decomposing at 194–196°. For analysis, the product was dried *in vacuo* at room temp.

over H_2SO_4 for 3 days. (Found: C, 33·3; H, 5·5; N, 11·7. $C_{10}H_{17}O_9N_3S$ requires C, 33·8; H, 4·8; N, 11·8%.) This was not a completely satisfactory analysis, but the identity of the compound was confirmed chromatographically. When run on paper with phenol–water, the compound gave a single spot, R_F 0·13, which reacted with ninhydrin but not with nitroprusside and cyanide nor with iodoplatinic acid. After being hydrolysed in 6N-HCl, the compound run in phenol–water in presence of NH_3 gave three ninhydrin-reacting spots corresponding to glutamic acid, cysteic acid and glycine.

L-*Cysteinylglycine*. This compound was prepared by partial hydrolysis of glutathione with N-HCl (Olson & Binkley, 1950).

SUMMARY

1. The three compounds γ-DL-glutamylglycylglycine, γ-DL-glutamyl-DL-alanylglycine and *S*(*N*-ethylsuccinimido)-L-cysteine have been synthesized.

2. From glutathione the following compounds have been prepared; *S*-methylglutathione, *S*(*N*-ethylsuccinimido)glutathione, γ-L-glutamyl-L-β-sulphoalanylglycine, L-cysteinylglycine and γ-L-glutamyl-L-alanylglycine, the last being obtained only in an impure state and in low yield.

3. On a paper chromatogram, phthalimido derivatives are detected by spraying with a reagent containing hydrazine hydrate. When the paper is dried, the phthalylhydrazide formed fluoresces strongly in ultraviolet light.

We are indebted to Dr Hazel Dobbie for the α-amino nitrogen analysis carried out on γ-DL-glutamylglycylglycine. We wish to thank the Medical Research Council for a studentship to one of us (N.A.M.) and The Distillers Co. (Biochemicals) Ltd. for a generous gift of glutathione.

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The Effects of some Analogues of Glutathione on the Glyoxalase System

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It is now established that the enzymic conversion of methylglyoxal into lactic acid takes place in two stages. First, methylglyoxal reacts with glutathione (GSH) to form *S*-lactylglutathione, the reaction being catalysed by an enzyme which Racker (1951) has called glyoxalase I. Secondly, this thioester is hydrolysed by another enzyme, glyoxalase II, giving lactic acid and regenerating GSH. The GSH therefore acts as a coenzyme for the overall reaction whereby methylglyoxal is transformed into lactic acid. Glyoxalase I and II correspond to the 'enzyme' and the 'factor' respectively of Hopkins & Morgan (1948).

Behrens (1941) has shown that, in the glyoxalase reaction, glutathione may be replaced as a coenzyme by either α-L-glutamyl-L-cysteinylglycine or β-L-aspartyl-L-cysteinylglycine, though both are less effective than GSH (γ-L-glutamyl-L-cysteinylglycine). Kögl & Akkerman (1946) found that γ-D-glutamyl-L-cysteinylglycine can also serve as a coenzyme for the glyoxalase system, but again it is less active than glutathione. Other substances tested as coenzymes but found ineffective include oxidized glutathione, cysteine, thioglycollic acid (Lohmann, 1932), γ-glutamylcysteine and cysteinylglycine (Woodward & Reinhart, 1942).

In the present work various analogues of GSH were tested as possible inhibitors of the glyoxalase reaction. Experiments with the complete glyoxalase system showed that certain of these analogues were indeed inhibitory but the simultaneous action of both glyoxalase I and II made quantitative interpretation of the results difficult. For this reason,

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most of the inhibition studies were carried out on glyoxalase I alone. GSH is a substrate for this enzyme, the other being methylglyoxal (or some other glyoxal derivative). The effect of any inhibitor which acts as an analogue of glutathione should therefore be primarily on glyoxalase I.

THEORETICAL

During the study of the effect of GSH analogues on glyoxalase I, it was found that certain of these compounds produced inhibition which was neither purely competitive nor purely non-competitive. A general equation of inhibition was therefore derived to cover competitive, non-competitive and intermediate types of inhibition (Matheson, 1954). Somewhat similar treatments have since come to our notice (Segal, Kachmar & Boyer, 1952; Botts & Morales, 1953) so that only the final equations will be given here.

In inhibition where the enzyme–substrate–inhibitor complex cannot break down to give the products of the reaction and the enzyme–substrate complex breaks down to yield the products of the reaction at a rate which is slow relative to the rate of formation of the complex, the initial velocity V is given by the general equation

$$\frac{V_{max.}}{V} = 1 + \frac{I}{K_l} + \frac{K_m}{S}\left(1 + \frac{I}{K_i}\right), \qquad (1)$$

where $V_{max.}$ is the velocity in the absence of inhibitor and all the enzyme is combined with the substrate, I the inhibitor concentration, S the substrate concentration, K_m the Michaelis constant, K_i the dissociation constant of the enzyme–inhibitor complex and K_l the dissociation constant of enzyme–substrate–inhibitor complex to enzyme–substrate complex and inhibitor.

This is the type of equation used by Lineweaver & Burke (1934). That of Hunter & Downs (1945) may be derived from it and is

$$I\frac{\alpha}{1-\alpha} = K_i\left(1 + \frac{S}{K_m}\right)\Big/\left(1 + \frac{K_i S}{K_l K_m}\right), \qquad (2)$$

where α, the fractional activity, is the ratio of the velocities of the reaction in presence and absence of the inhibitor at a given substrate concentration S.

If in these equations K_l is infinite (which implies that the enzyme–substrate–inhibitor complex cannot exist), the well-known equations for competitive inhibition are obtained, whilst if $K_l = K_i$ the equations for non-competitive inhibition result, as may be readily verified. The situation where the substrate and the inhibitor each reduce but do not abolish the affinity of the enzyme for the other is represented by taking values of K_l between K_i and infinity. This case may be called 'intermediate' inhibition.

In the present study of the effects of inhibitors, the form of equation used by Hunter & Downs has advantages over that of Lineweaver & Burke. First, the Hunter & Downs method requires the experimental determination of the ratio of the velocities of the inhibited to the uninhibited reaction at various concentrations of inhibitor. The two velocities can be conveniently measured simultaneously in a spectrophotometer and unavoidable variations in the experimental conditions affect each velocity similarly and so tend to cancel out. Secondly, all data from experiments with different concentrations of inhibitor can be used to define a single line from which deductions can be made about the type of inhibition.

In Fig. 1 plots of the Hunter & Downs type are shown for $K_l = \infty$, $K_l = K_i$ and for two intermediate values of K_l, K_i and K_m being constant in all four cases. Competitive inhibition ($K_l = \infty$) gives a single straight line cutting the $I\alpha/(1-\alpha)$ axis at K_i and the S axis at $-K_m$. Non-competitive inhibition ($K_l = K_i$) is represented by another straight line which is parallel to the S axis and cuts the $I\alpha/(1-\alpha)$ axis at K_i. For an intermediate value of K_l, the plot of $I\alpha/(1-\alpha)$ against S is not linear but is a curve cutting the vertical axis at K_i and the

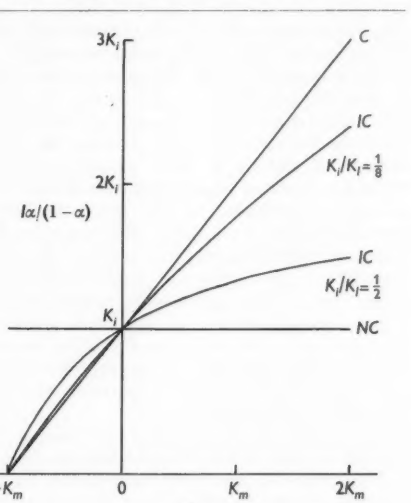


Fig. 1. Plot of $I\alpha/(1-\alpha)$ against S in different types of inhibition. *C*, Competitive inhibition: single straight line which cuts $I\alpha/(1-\alpha)$ axis at K_i and S axis at $-K_m$. *NC*, Non-competitive inhibition: single straight line which is parallel to S axis and cuts $I\alpha/(1-\alpha)$ axis at K_i. *IC*, Intermediate case: all lines are curved and cut $I\alpha/(1-\alpha)$ axis at K_i and S axis at $-K_m$; each value of K_i/K_l produces one curve, those shown being for $K_i/K_l = \frac{1}{2}$ and $K_i/K_l = \frac{1}{8}$.

horizontal axis at $-K_m$. The degree of curvature is determined by the ratio of K_i to K_l.

Thus in the Hunter & Downs plot there are three possibilities. The graph may be a straight line parallel to the S axis. With this the inhibition is of the non-competitive type and the value of K_i is given by the point of intersection with the $I\alpha/(1-\alpha)$ axis. Secondly, the plot may be a straight line inclined to the S axis and cutting it at $S=-K_m$. Here the inhibition is competitive and K_i is given by the intersection of the line and the $I\alpha/(1-\alpha)$ axis. In the third case, the observed points lie on a curve which, however, will not deviate greatly from linearity over the relatively limited range of substrate used. If the best-fitting straight line is drawn through these points it will be again inclined to the S axis but will cut it at values of $-S$ appreciably greater than K_m; in this case inhibition is of the intermediate type.

The point at which the best-fitting straight line cuts the $I\alpha/(1-\alpha)$ axis will not in general coincide with the intersection of the curve with this axis, which is at K_i. However, it can be shown that the tangent to the curve at the point where S equals K_m cuts the vertical axis at $K_i(1+3K_i/K_l)/(1+K_i/K_l)^2$. This expression can be shown to have a maximum value for a fixed K_i when K_l equals $3K_i$. This maximum value is therefore $9K_i/8$. The tangent to the curve at any point between S equals 0 and S equals K_m must therefore cut the ordinate between K_i and $9K_i/8$. It follows that the straight line best fitting a series of points on the curve, for values of S between 0 and K_m, must cut the ordinate between K_i and $9K_i/8$. If the concentration of substrate in a series of experiments does not exceed K_m then the intersection of the best-fitting straight line with the $I\alpha/(1-\alpha)$ axis will give a value which does not differ from the true value of K_i by more than 12·5 %, and which will usually be much closer than that. This agreement demonstrates the approximate linearity of the curve between S equals 0 and S equals K_m and justifies the use of observations over this range for distinguishing between the various types of inhibition according to the method here suggested.

It is well known (Jowett & Quastel, 1933; Schubert, 1935) that methylglyoxal and GSH react non-enzymically to yield an adduct, probably according to the equation

$$CH_3.CO.CHO + GSH \rightleftharpoons CH_3.CO.CH(OH).SG. \quad (3)$$

It is not known whether glyoxalase I acts on the adduct or on free methylglyoxal and GSH. In the latter case the enzyme would have two substrates. A mathematical treatment applicable to the case of an enzyme acting on two substrates was therefore developed (Matheson, 1954). At any fixed concentration of the second substrate, the resulting equations are equivalent to those already given provided it is assumed that combination of the enzyme with the second substrate does not affect its affinity for either the first substrate or the inhibitor. Whether the enzyme combines with the adduct or its free components, the same type of equation will be obtained to describe the dependence of the velocity on the concentration of the adduct or of the GSH as the case may be.

There is evidence, as will be seen below, that the equilibrium constant of the above reaction is of the order of 10^{-2} M. It can be calculated that within the range of concentrations of methylglyoxal and GSH used, only a small proportion of the methylglyoxal and of the glutathione exist in the combined form, and therefore, provided that the concentration of methylglyoxal is kept constant, the amounts of adduct and of free GSH are approximately proportional to the total GSH concentration. From this result and the conclusions reached in the preceding paragraph it may be inferred that the dependence of the velocity on the total GSH concentration will follow the same type of equation for constant methylglyoxal concentration whether the enzyme reacts with the adduct or independently with the two components.

It is convenient in the first instance to give the values of K_i and K_m in terms of the total GSH concentration. The real values of K_i and K_m should be expressed in terms of concentration of free GSH or of adduct, according to which theory is correct. It will be clear that for constant methylglyoxal concentration this figure will be proportional to the value deduced from the total GSH concentration. It will be seen later that it is probable that glyoxalase I acts on free GSH.

EXPERIMENTAL

Materials

Methylglyoxal. A commercial 30% (w/v) solution was diluted to about 10% and distilled twice under reduced pressure, the fraction boiling below 50° at 20 mm. Hg being collected. The distillate was standardized by the method of Friedemann (1927), which is accurate to ±1% (Woo & Chang, 1945). This solution, which also contained lactic acid, was stored at 0° and was stable over a period of several months.

Glutathione. The material used was a preparation of over 99% purity [The Distillers Co. (Biochemicals) Ltd.]. The solutions were prepared immediately before use and brought to pH 6·6 with NaOH.

Glutathione analogues. The preparation of these compounds is described in a previous paper (Kermack & Matheson, 1957). Neutral solutions of the required concentration were stored at 0° and fresh solutions were made every few days. Where the analogue contained a sulphydryl group, a solution was prepared immediately before use.

Phosphate buffers. These were prepared by mixing stock *M* solutions of KH_2PO_4 and K_2HPO_4 in the required proportions and diluting as necessary.

Serum albumin. A crude commercial preparation of serum albumin (B.D.H.) was purified by fractionation with $(NH_4)_2SO_4$, the material precipitated between 50 and 100% saturation being taken. The concentration of albumin was determined by micro-Kjeldahl analysis after removal of $(NH_4)_2SO_4$ by thorough dialysis.

Glyoxalase I and II. The enzymes were prepared from yeast and from ox liver respectively by the methods of Racker (1951). The glyoxalase I preparation was taken to step 8 and the glyoxalase II preparation to step 4. Both enzymes were stored at $-15°$. Glyoxalase I was very stable in concentrated solution, no change in activity being found on storage for a year at $-15°$. When diluted, however, it was very unstable but could be stabilized by serum albumin (Racker, 1951). Solutions for kinetic studies were made by diluting the stock solution 1:500 with 0·1 M potassium phosphate buffer, pH 7·4, containing 100 μg. of serum albumin/ml. Glyoxalase II was much less stable; even in concentrated solution it lost all its activity within a few weeks. The stock solution itself was used in manometric studies of the complete glyoxalase system.

Methods

Measurement of the activity of the complete glyoxalase system. These measurements were carried out in a standard Warburg manometric apparatus. Since the potential inhibitors used were analogues of GSH, which is in effect a substrate of glyoxalase I, it was desirable that this enzyme should be the rate-limiting factor. In practice this condition was never completely fulfilled, since sufficiently active preparations of glyoxalase II were not obtained. All the Warburg flasks contained $NaHCO_3$, GSH, methylglyoxal, glyoxalase I and II and certain flasks also contained the analogue being studied. Half the $NaHCO_3$ and all the methylglyoxal were present in the side arm. The flasks were swept out with N_2+CO_2 (95:5, v/v) for 10 min., then equilibrated in a bath at 25° for a further 10 min. The side-arm contents were added and the reaction was allowed to proceed for 5 min. before the taps were closed, to eliminate errors due to evolution of gas produced by chemical changes on mixing. Readings were taken on closing the taps and subsequently at 5 min. intervals. The velocity of the reaction was linear over the whole period of measurement.

Measurement of the activity of glyoxalase I. Racker's (1951) observation that *S*-lactylglutathione, which is formed by the action of glyoxalase I, has a high optical density at 240 mμ. led him to develop a spectrophotometric method suitable for the measurement of glyoxalase I activity. The rate of reaction was measured by following the increase in optical density at 240 mμ. by means of a Unicam SP. 500 quartz spectrophotometer. Into each of two 1 cm. quartz cells were pipetted phosphate buffer, pH 6·6 (final concn. $3·3 \times 10^{-2}$ M), methylglyoxal and GSH. The analogue under study was added to one cell; to each cell water was added to give a total volume of 3·0 ml., after addition of enzyme. The cells were read against a blank containing water and buffer. After the initial increase in readings had stopped, 0·1 ml. of diluted glyoxalase I solution was added to each cell, the moment of mixing being taken as zero. The cells were replaced in the instrument as rapidly as possible and readings were made in quick succession, the exact time of each reading being noted. The readings were plotted against time and the initial reading was obtained by extrapolation. During the first 5 or 10 min. the curves were practically linear at the enzyme and substrate concentrations used in experiments on inhibition, so the rate of reaction was found by dividing the change in optical density by the time. Room temp. was held at $20 \pm 1°$: the temperature of the solutions in the cells did not change during the period of measurement.

RESULTS

Experiments with the complete glyoxalase system

A number of experiments were carried out in the Warburg apparatus to test the effect of analogues of glutathione on the complete glyoxalase system. No great importance can be attached to the quantitative aspect of these experiments, but several of the compounds proved to be inhibitory. The results for some of the analogues are shown in Fig. 2.

Experiments with glyoxalase I

Non-enzymic reactions. As a preliminary the non-enzymic reactions were studied spectrophotometrically. While a solution of methylglyoxal

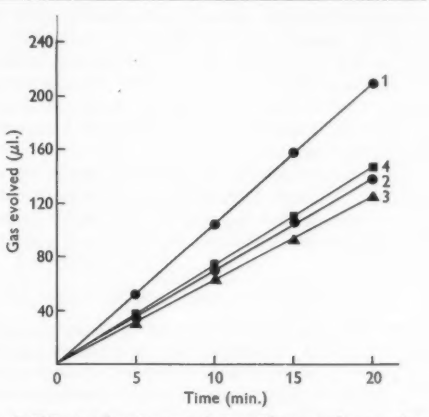


Fig. 2. Effects of analogues of glutathione on the complete glyoxalase system in the Warburg apparatus. All flasks contained potassium bicarbonate, $4·0 \times 10^{-2}$ M, methylglyoxal, $2·2 \times 10^{-2}$ M, glutathione $1·62 \times 10^{-3}$ M, glyoxalase I, 0·05 ml. of 1:100 stock solution, glyoxalase II, 0·3 ml. of stock solution. Flask 1 was an uninhibited control; flasks 2–4 contained *S*-methylglutathione, $6·5 \times 10^{-2}$ M, *S*(*N*-ethylsuccinimido)glutathione, $6·5 \times 10^{-2}$ M, and *S*-methylcysteine, 16×10^{-2} M, respectively. All figures are of final concentration after tipping in: the side arm contained half the bicarbonate and all the methylglyoxal. The pH of the flask contents was 7·6 and reaction was carried out at 25°; the taps were closed 5 min. after tipping in. The final volume was 2 ml.

($2{\cdot}16 \times 10^{-3}$ M) in phosphate buffer ($3{\cdot}3 \times 10^{-2}$ M) at pH 6·6 did not change in optical density at 240 mμ. over a period of 5 hr., a solution of GSH ($2{\cdot}16 \times 10^{-3}$ M) slowly increased in absorption after an initial lag period of about 15 min. The increase was approximately linear, being about $2{\cdot}5 \times 10^{-2}$/hr. over the 5 hr. period. This slow increase may be due to oxidation of the sulphydryl group to a disulphide. The fact that the intensity of the colour produced in the nitroprusside test for sulphydryl groups fell, as the optical density rose, supports this view.

The behaviour of a mixture of glutathione and methylglyoxal (each $2{\cdot}16 \times 10^{-3}$ M) in phosphate buffer was quite different from that of the separate components. On mixing, there was a relatively rapid increase in optical density (0·14) during the first 60–70 min., followed by an increase similar to that observed with GSH alone but at an even slower rate ($1{\cdot}3 \times 10^{-2}$/hr.).

It is known that methylglyoxal and GSH react non-enzymically to form a labile addition compound (Jowett & Quastel, 1933; Schubert, 1935). The relative amount of free and of combined GSH in the presence of methylglyoxal was determined by Platt & Schroeder (1934) by rapid titration of free sulphydryl groups. From their results it is possible to deduce that the dissociation constant K of the addition compound is probably of the order of 10^{-2} M, but the values of K calculated from their various experiments extend over a considerable range. This lack of constancy of K may be due to progressive dissociation of the addition compound during the titration.

A new estimate of K has been made by the following method. Various amounts of glutathione were mixed with a solution of methylglyoxal and the change in optical density accompanying the reaction was measured. For each mole of complex formed, one mole of GSH and one mole of methylglyoxal would disappear. Thus if n be the number of moles of the complex formed, and m_1, m_2 and m_3 be the molecular absorption coefficients of the complex, methylglyoxal and GSH respectively, the change in optical density will be proportional to $n(m_1 - m_2 - m_3)$, that is, to n. Now if the initial concentration of methylglyoxal (C) is kept constant and several GSH concentrations are used, the concentration of the complex formed (q) will depend on the concentration of GSH (S) according to the equation

$$\frac{(S-q)\,(C-q)}{q} = K. \qquad (4)$$

From this, assuming some particular value of K, we can calculate the concentrations of the complex, q_1, q_2, q_3, etc., for the concentrations of GSH, S_1, S_2, S_3, etc. The values of q are, however, proportional to the corresponding values of n, that is, they should be proportional to the measured changes in optical density. Thus the required value of K will be that for which this condition is satisfied, and from Table 1 this value of K is $10^{-2{\cdot}3}$ M. The advantage of this method is that it depends on physical measurements which do not interfere with the equilibrium (as titration of the free SH groups may do) and that independent knowledge of the absorption coefficient of the complex is not necessary. It is not claimed that this method is very accurate as the slow increase in optical density in a solution of GSH is an unavoidable complication. For the purpose of the present work only an approximate value of K was required.

Enzymic reactions. Action of the enzyme on the addition compound and on its components. Glyoxalase I may act on free GSH and free methylglyoxal or on their addition compound. The

Table 1. *Evaluation of the dissociation constant K of the methylglyoxal–GSH complex*

All cells contained buffer and water to 3·0 ml. final vol.; cells 2, 3 and 4 contained methylglyoxal, 2·16 mM, and varying amounts of GSH. Readings were made before addition of GSH, immediately afterwards, and at frequent intervals for the next 70 min., by which time the increase in optical density had practically stopped. The initial reading on the mixture of GSH and methylglyoxal was obtained by extrapolating the curves back to zero time.

	Cell		
	2	3	4
Concn. of GSH (mM)	1·08	2·16	4·32
Change in log I_0/I	0·086	0·155	0·255
Ratio of changes in log I_0/I	1·00	1·80	2·97
Calc. concn. of addition compound (mM), when			
$K = 10^{-2}$	0·18	0·34	0·59
$K = 10^{-3}$	0·65	1·11	1·58
$K = 10^{-2{\cdot}3}$	0·29	0·53	0·88
Ratio of calc. concn. of addition compound (mM), when			
$K = 10^{-2}$	1·00	1·89	3·28
$K = 10^{-3}$	1·00	1·71	2·43
$K = 10^{-2{\cdot}3}$	1·00	1·83	3·03

velocity of the reaction might therefore depend on whether the equilibrium concentration of the intermediate compound had been reached before the enzyme was added.

This possibility was studied by filling two cells with buffer and methylglyoxal. GSH was added to one cell and after the relatively rapid, non-enzymic, increase in optical density had ceased, a similar amount of GSH was added to the other cell. To each cell was immediately added a solution of the enzyme and the rate of increase in optical density was determined. The results are shown in Fig. 3. The initial rate was slightly faster in the cell to which the enzyme had been added immediately after the components were mixed. In this cell there was little or no addition compound at the beginning of the enzymic reaction, whereas in the other cell the concentration of the addition compound was much greater and yet the reaction went more slowly. This definitely suggests that the enzyme did not act on the addition compound but on the free GSH and methylglyoxal. The rather greater initial rate of reaction in the cell containing the free components may be accounted for by the fact that the increment in optical density when the components change into the enzymically formed compound is greater than that associated with the change of the addition compound into the enzymically formed compound. The changes in optical density/mole can be shown to be about 5 % greater in the former than in the latter case. This is less than the difference in the initial reaction rates shown in Fig. 3, but other factors, such as the slightly higher concentrations of the free components where equilibrium has not been allowed to occur and the initial tendency for the chemical addition compound to form, probably account for the rest of the difference. In subsequent work, to ensure standard conditions time was allowed for equilibrium to be reached between the addition compound and the free components before the enzyme was added. It should be noted that the non-enzymic changes in optical density are small compared to enzymic ones at the same substrate concentrations.

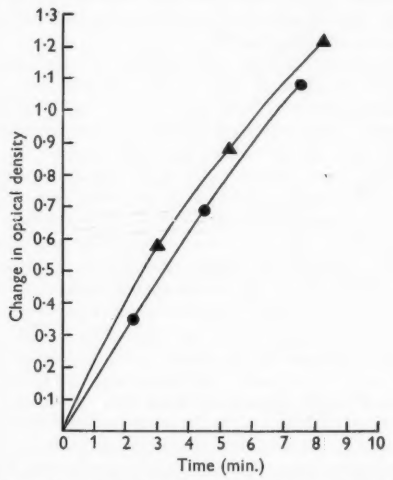


Fig. 3. Rate of the glyoxalase I reaction on free GSH and free methylglyoxal and on their addition compound. All cells contained phosphate buffer, $3{\cdot}3 \times 10^{-2}$ M at pH 6·6, and water to a final volume of 3 ml. Cells 2 and 3 contained GSH, $1{\cdot}08 \times 10^{-3}$ M, and methylglyoxal, $2{\cdot}16 \times 10^{-3}$ M, concn. after addition of enzyme in each case. In cell 2 (●) methylglyoxal was added initially but in cell 3 (▲) it was not added till the change in optical density in cell 2, due to the formation of addition compound, had stopped. Glyoxalase I (0·1 ml. of 1:200 stock solution) was then added immediately to both cells, and the rate of reaction measured spectrophotometrically.

Effect of substrate concentration on reaction velocity: determination of K_m. The effect on reaction velocity of varying the concentration of GSH was studied with different concentrations of methylglyoxal. The increase in optical density was plotted against time and the rate of reaction was found from the initial linear portion of the curve. When the reciprocals of velocity were plotted against those of GSH concentration, the points for a constant methylglyoxal concentration fell on a line which was straight except near the $1/V$ axis, where it curved to run horizontally. This corresponded to the fact that the velocity of the reaction attained a limiting value when the concentration of GSH reached that of methylglyoxal. Further increases in the GSH concentration did not produce any increases in the reaction velocity within the limits of experimental error.

Two such lines, each for a constant concentration of methylglyoxal, are shown in Fig. 4. It will be seen that the linear portions of the lines, when extrapolated, meet the $1/S$ axis at $-1{\cdot}82 \times 10^3$ and $-1{\cdot}58 \times 10^3$, giving values of K_m of $0{\cdot}55 \times 10^{-3}$ M and $0{\cdot}63 \times 10^{-3}$ M for methylglyoxal concentrations of $1{\cdot}08 \times 10^{-3}$ M and $2{\cdot}16 \times 10^{-3}$ M respectively. The average of these estimates is approximately $0{\cdot}6 \times 10^{-3}$ M, and this is taken as the value of K_m calculated from S, the total GSH concentration.

It appears from the previous section that the enzyme acts on free GSH, and therefore it would seem theoretically preferable to calculate the concentration of free GSH (S') in each experiment from equation (4) with $10^{-2{\cdot}3}$ M, i.e. 5×10^{-3} M, as the value of K. The effect of this modification depends on the concentration of methylglyoxal (C). If S, the total GSH concentration, is less than C so

that q is much less than C, equation (4) may be written

$$\frac{C(S-q)}{q}=K, \tag{5}$$

or

$$\frac{S'}{S}=\frac{S-q}{S}=\frac{K}{K+C},$$

i.e.

$$\frac{\text{free GSH}}{\text{total GSH}}=\frac{K}{K+C}. \tag{6}$$

This expression is approximately equal to $\frac{5}{6}$ when C is $1{\cdot}08\times10^{-3}$ M and $\frac{5}{7}$ when C is $2{\cdot}16\times10^{-3}$ M. If in Fig. 4 $1/V$ had been plotted against $1/S'$ instead of against $1/S$, the abscissae of all the observed points would have been increased by $\frac{1}{5}$ and $\frac{2}{5}$ in the respective cases. The straight lines best fitting the points where the glutathione concentration is not greater than that of methylglyoxal are shown as two broken lines in Fig. 4, which on extrapolation meet the substrate axis at $-1/S'$ equals $2{\cdot}20\times10^{3}$ M^{-1} and $2{\cdot}21\times10^{3}$ M^{-1} for methylglyoxal concentrations of $1{\cdot}08\times10^{-3}$ M and $2{\cdot}16\times10^{-3}$ M respectively. This close agreement lends further support to the view that the enzyme acts on free GSH. It further suggests that over the range of concentrations used, the underlying assumptions of the theory are valid and the methylglyoxal concentration does not affect the GSH–enzyme equilibrium. The Michaelis constant K'_m for the equilibrium: enzyme + free GSH $\rightleftharpoons$ enzyme–GSH, has the value $0{\cdot}46\times10^{-3}$ M.

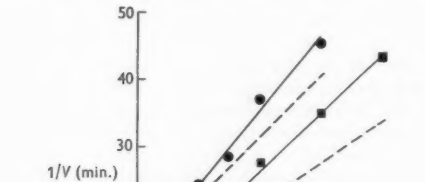


Fig. 4. Plot of $1/V$ against $1/S$ for two methylglyoxal concentrations. The concentrations of methylglyoxal used were $1{\cdot}08\times10^{-3}$ M (●), and $2{\cdot}16\times10^{-3}$ M (■). At each methylglyoxal concentration the velocity, V, was measured at a series of glutathione concentrations, S. The solid lines are those which best fit the points where S is equal to or less than the methylglyoxal concentration. The broken lines are obtained if the calculated free GSH concn. are used instead of the total GSH concn.

Effects of analogues of glutathione on the glyoxalase I reaction. The initial velocity of the reaction in cells with and without the inhibitor was determined at a series of inhibitor and substrate concentrations. From the fractional activity α, which is the ratio of the velocities of the inhibited to the uninhibited reaction, $I\alpha/(1-\alpha)$ was calculated and then plotted against substrate concentration. The 'best' straight line was fitted to the experimental points by the method of least squares and extrapolated to meet the substrate axis, where possible.

As already indicated, these lines should cut the S axis at $S=-K_m$ if inhibition is competitive and at infinity if non-competitive. If they are cut at some other value of S then the inhibition is of an intermediate type.

The graphs of $I\alpha/(1-\alpha)$ against S for various analogues of glutathione and allied compounds are shown in Figs. 5–7. These experiments are summarized in Table 2. In this table K_i has been obtained as the value of $I\alpha/(1-\alpha)$ at which the line best fitting the experimental points cuts the ordinate axis. It has been shown in the Theoretical section that in intermediate-type inhibition the intercept differs from the true value of K_i by less than 12·5 %, provided that the substrate concentrations are less than K_m.

Since the compounds tested had appreciable optical densities at 240 mμ., a limit was set to the concentrations which could be used without affecting the accuracy of the spectrophotometer readings. As a result, for weak inhibitors (K_i greater than about 10^{-2}) only small degrees of inhibition could be produced; the value of $1-\alpha$ was therefore small and the error in $\alpha/(1-\alpha)$ relatively large. Thus with such weak inhibitors, points may show a considerable degree of scatter in comparison with those for inhibition by stronger ones.

Experiments were also carried out with L-cysteine and L-cysteinylglycine as inhibitors. With low analogue concentrations no inhibition was observed. As the concentration of the analogue approached that of methylglyoxal a large increase in optical density occurred before the addition of the enzyme, and the velocity of the subsequent enzymic reaction was diminished (Fig. 8). This suggests that these two compounds react non-enzymically with methylglyoxal and prevent it from taking part in the enzymic reaction.

DISCUSSION

The postulated mechanism of the glyoxalase reaction involves the formation of *S*-lactylglutathione by glyoxalase I. Evidence in support of this conclusion was first obtained by Racker (1951), who by using glyoxalase I free from glyoxalase II was able to prepare the intermediate compound and

showed that its formation was accompanied by a marked increase in absorption at 240 mμ. This characteristic light absorption disappeared when the compound was broken down by glyoxalase II. Thioesters in general show a characteristic absorption peak in this neighbourhood (Racker & Krimsky, 1952; Burton & Stadtman, 1953). Strecker (1954) showed that glyoxalase II can replace brain thioesterases in the hydrolysis of certain thioesters, in conformity with the view that glyoxalase II is a thioesterase. The identity of the thioester has been confirmed by Racker (1954) who has shown that *S*-lactylglutathione prepared synthetically (Wieland & Koppe, 1953) is hydrolysed by glyoxalase II at approximately the same rate as the enzymically prepared intermediate.

With regard to the kinetics of the reaction catalysed by glyoxalase I, it has been shown in the present work that when $1/V$ is plotted against $1/S$, the curve obtained is approximately linear for sufficiently small values of S but departs from

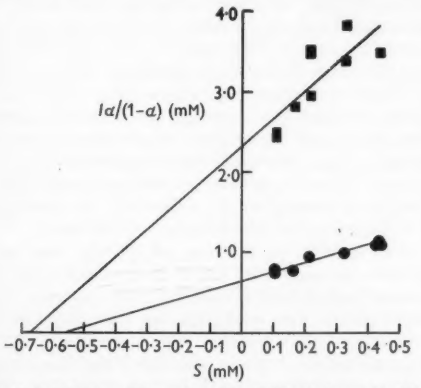


Fig. 5. Inhibition of glyoxalase I by *S*(*N*-ethylsuccinimido)-glutathione (●), and by *S*-methylglutathione (■): plot of $I\alpha/(1-\alpha)$ against S; α is determined as the ratio of inhibited velocity to uninhibited velocity with the same concentration of substrate S, over a period of 5 min.

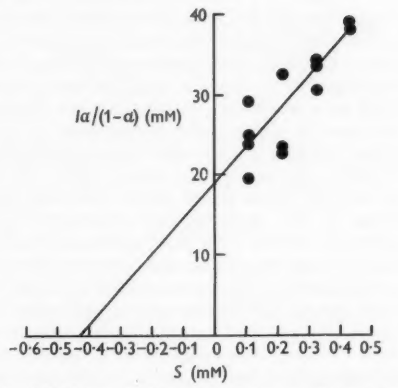


Fig. 6. Inhibition of glyoxalase I by γ-L-glutamyl-L-β-sulphoalanylglycine.

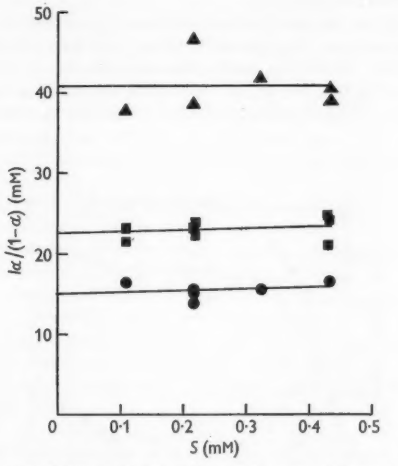


Fig. 7. Inhibition of glyoxalase I by γ-DL-glutamyl-DL-alanylglycine (●), by γ-DL-glutamylglycylglycine (▲) and by *S*-methyl-L-cysteine (■).

Table 2. *Action of GSH analogues on the glyoxalase I reaction*

Values of K_i were obtained from experimental observations of the type illustrated in Figs. 5–7, by the method described in the Theoretical section.

Inhibitor	K_i (mM)	Apparent K_m (mM)	Type of inhibition
S(*N*-Ethylsuccinimido)glutathione	0·64	0·55	Competitive
S-Methylglutathione	2·3	0·67	Competitive
γ-DL-Glutamyl-DL-alanylglycine	15·1	8·3	Intermediate, largely non-competitive
γ-L-Glutamyl-L-β-sulphoalanylglycine	18·7	0·43	Competitive
γ-DL-Glutamylglycylglycine	40·9	∞	Non-competitive
S-Methyl-L-cysteine	22·5	9·3	Intermediate, largely non-competitive
S(*N*-Ethylsuccinimido)-L-cysteine	—	—	None
Glutamic acid	—	—	None
Glycine	—	—	None

linearity near the $1/V$ axis. This corresponds to the fact that the velocity of the reaction increases with increase of GSH concentration according to the Michaelis–Menten equation until the concentration of GSH approaches that of methylglyoxal. Once the concentration of the latter is exceeded, however, further increases in the GSH concentration have little effect on the reaction velocity. The converse does not hold since at any GSH concentration an increase in the methylglyoxal concentration increases the velocity. No explanation which will fit all the facts can be advanced at present. However, the experiments with enzyme inhibitors have been carried out at GSH concentrations well below that of methylglyoxal, that is, on the linear portion of the curves. Under these conditions it would seem justifiable to treat the enzyme in equilibrium with a fixed concentration of methylglyoxal as if it were a simple enzyme reacting with GSH at these low concentrations according to the usual Michaelis–Menten theory.

If, as appears probable from the considerations adduced in the Results section, it is the free GSH which combines with the enzyme, it is more appropriate to use the calculated concentrations of free GSH rather than those of total GSH in working out the kinetics of the reaction. This change does not significantly alter the nature of the graphs. The chief result is to lower the values of K_m calculated from the two methylglyoxal concentrations and to bring them into very close agreement.

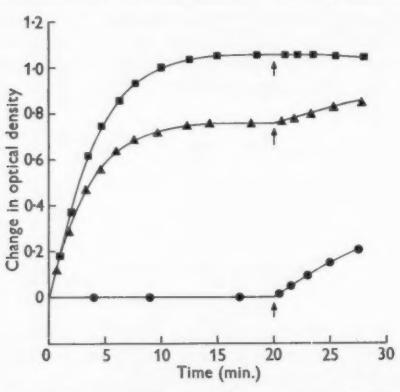


Fig. 8. Action of cysteine on methylglyoxal: inhibition of glyoxalase I. All cells contained buffer and water to bring volume to 3·0 ml. after addition of the enzyme. In addition, cells 2 (●), 3 (▲) and 4 (■) contained methylglyoxal, $2{\cdot}16 \times 10^{-3}$ M, and GSH, $0{\cdot}216 \times 10^{-3}$ M, and cells 3 and 4 contained cysteine, $2{\cdot}16 \times 10^{-3}$ M and $4{\cdot}32 \times 10^{-3}$ M respectively. These concentrations are those attained after the addition of the enzyme. The cells were equilibrated for 10 min. before cysteine was added. Measurements began immediately this addition was made, by using cell 1 as blank and extrapolating to zero time to find the initial reading. After 20 min. an almost steady reading had been reached in all cells. To each was added 0·1 ml. of glyoxalase I (stock solution diluted 1:500) and the subsequent enzymic reactions were followed.

In the treatment of results from the experiments with inhibitors it has been considered best to employ total GSH concentration, as this involves fewer assumptions about the methylglyoxal–GSH equilibrium. In all these experiments the same concentration of methylglyoxal has been used so that in accordance with equation (6), the ratio of free to total GSH would remain constant. This implies that all the relevant graphs would merely be altered in scale as far as the abscissae are concerned and none of the conclusions would be affected.

The discussion in the theoretical section serves to underline the fact, generally recognized by workers in the field, that the enzyme kinetics of an inhibited reaction may not correspond to either a purely competitive or purely non-competitive type. Owing to the inevitable experimental error it is often difficult to decide whether the observed departure from one or other of these two types is significant or not. The test we have applied is to plot the value of $I\alpha/(1-\alpha)$ against S, the substrate concentration, where S is less than K_m. If the best-fitting straight line through the observed points cuts the S axis at or close to the point where S equals $-K_m$, the inhibition is presumably competitive. Non-competitive inhibition is suggested if this line is parallel to the S axis and intermediate lines suggest intermediate types of inhibition.

For purely competitive or purely non-competitive inhibition the value of K_i is given by the intersection of the line and the $I\alpha/(1-\alpha)$ axis. For intermediate-type inhibition the true value of K_i will, in general, be less than the value of $I\alpha/(1-\alpha)$ at this intersection, but as was shown in the Theoretical section the difference will be less than $K_i/8$. The value of $I\alpha/(1-\alpha)$ at the point of intersection is therefore a reasonable approximation to K_i.

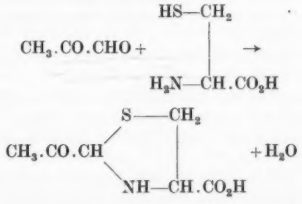
In considering the action of the various compounds used in the inhibition experiments, we find that the most active of the tripeptides listed in Table 2 is *S*(*N*-ethylsuccinimido)glutathione, followed by *S*-methylglutathione. Both these compounds act in a purely competitive manner as also does γ-L-glutamyl-L-β-sulphoalanylglycine. γ-DL-Glutamyl-DL-alanylglycine has a fairly high K_i value and therefore would be classed as a weak inhibitor, but it should be remembered that the synthetic compound is a mixture of four stereoisomeric forms. If only one of these forms, the LL, were active the value of K_i would only be a quarter of that found, so that this stereoisomeric form would be almost comparable in activity with *S*-methylglutathione, though its inhibition is of a

mixed type. γ-DL-Glutamylglycylglycine had a very low activity of the non-competitive type. Here it is possible that only one of the two stereoisomers is active, but, even if allowances were made for this, the compound has only low inhibitory power. It would, of course, have been preferable to have synthesized the pure optical isomers and used them in these inhibition experiments but the time available did not permit this extension of the work.

Of the group of analogues which are not tripeptides only *S*-methyl-L-cysteine was definitely inhibitory and showed an intermediate, largely non-competitive, type of inhibition. It is rather curious that the *N*-ethylmaleimide adduct of GSH is more effective than *S*-methylglutathione, and *S*-methylcysteine is inhibitory and the *N*-ethylmaleimide adduct of cysteine is not.

Thus the compounds which are tripeptides differing from GSH only in the $-CH_2.SH$ side chain are the most effective inhibitors. Those compounds which are not tripeptides are either weak inhibitors or are without effect.

The behaviour of two of the analogues, not possessing the tripeptide structure, calls for further comment: these are L-cysteine and L-cysteinylglycine. In the presence of methylglyoxal both compounds undergo a reaction which is accompanied by an increase in optical density at 240 mμ. It is believed that the formation of a thiazolidine ring occurs with the elimination of water (Schubert, 1935, 1936).



In the range of concentrations used, methylglyoxal was not completely combined unless a considerable excess of aminothiol was present, for increasing the concentration of the latter beyond that of methylglyoxal produced a further increase in the optical density in the absence of enzyme. The removal of methylglyoxal inhibited the enzymic reaction with GSH, and when two molecules of aminothiol were present for each molecule of methylglyoxal, the enzymic reaction ceased.

Racker (1951) suggested that oxidized glutathione could be estimated by the glyoxalase reaction in the Warburg apparatus, after its reduction with excess cysteine. It is clear that this method will only be satisfactory provided that sufficient methylglyoxal remains, after addition of the cysteine, to allow the reaction to proceed at maximum velocity.

Fodor, Miller & Waelsch (1952, 1953) have reported that estimations of GSH by the glyoxalase system in the Warburg apparatus are inaccurate in the presence of cysteinylglycine. They believed that this was due to competitive inhibition. It appears likely that the inhibition was due to combination of the peptide with methylglyoxal rather than to direct competition between cysteinylglycine and GSH.

SUMMARY

1. Some glutathione analogues were tested on the complete glyoxalase system (glyoxalase I plus glyoxalase II) and shown to be inhibitory. No quantitative deductions could be made because of the complexity of the system.

2. The non-enzymic reaction between glutathione and methylglyoxal in aqueous solution involved a small increase in optical density which could be used to follow the reaction. The equilibrium constant was shown to be approximately $10^{-2.3}$ M.

3. The variation in the velocity of the enzymic reaction with glutathione concentration was studied at two different concentrations of methylglyoxal. The results suggest that the enzyme combines with free GSH and not with the GSH combined with methylglyoxal in the non-enzymic reaction, and that the Michaelis constant for the equilibrium is $0{\cdot}46 \times 10^{-3}$ M.

4. The effect of analogues of glutathione on the glyoxalase I reaction was studied and some were found to be inhibitory. The stability constants of the enzyme–inhibitor complexes (K_i) were calculated and the nature of the inhibition was investigated.

5. The relationship between the structure of the analogues and the degree and type of inhibition they produced is discussed in the light of the mathematical theory of enzyme inhibition briefly outlined.

6. A non-enzymic reaction of L-cysteine and of L-cysteinylglycine with methylglyoxal caused a large increase in optical density. It differed from the reaction of glutathione and methylglyoxal and probably involved the formation of a thiazolidine ring.

We wish to thank the Medical Research Council for a studentship to one of us (N.A.M.) and The Distillers Co. (Biochemicals) Ltd. for a generous gift of glutathione.

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The Conversion of Dimefox into an Anticholinesterase by Rat Liver *in vitro*

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The organic phosphorus insecticides schradan (bisdimethylaminophosphonous anhydride) and parathion (*OO*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) have been shown to be converted by mammals *in vivo*, and by liver tissue *in vitro*, into powerful anticholinesterases (see, e.g. Gardiner & Kilby, 1950*a*, *b*; Dubois, Doull & Coon, 1950; Diggle & Gage, 1951; Aldridge & Barnes, 1952; Casida, Allen & Stahmann, 1952, 1954; Gage, 1953; Metcalf & March, 1953; Davison, 1954, 1955).

Dimefox (bisdimethylaminofluorophosphine oxide), another insecticide, was claimed by Aldridge & Barnes (1952) to be converted into a more active inhibitor *in vivo*. High concentrations were required to inhibit rabbit-erythrocyte cholinesterase *in vitro* as compared with those calculated to occur *in vivo* after injection of dimefox. They reported that about 7×10^{-2} M dimefox caused 50% inhibition of rat-brain cholinesterase, and that rat-liver slices converted it into a more active but unstable anticholinesterase. Okinaka, Doull, Coon & Dubois (1954), however, found that 4×10^{-5} M dimefox *in vitro* caused 50% inhibition of rat-brain cholinesterase in 15 min., and that rat-liver slices reduced the anticholinesterase activity aerobically or anaerobically.

This paper presents further studies on the mode of action and metabolism of dimefox in mammals. The work was undertaken as part of a programme of investigation of the toxicology of dimefox, the systemic insecticidal properties of which are utilized to combat insect infestation on hops, sugar beet and other crops.

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MATERIALS AND METHODS

Dimefox, b.p. 205° at 760 mm., 96° at 18 mm., was prepared by fractional distillation of Hanane (Fisons Pest Control Ltd.) and was 98·6% pure by chemical assay. Diphosphopyridine nucleotide (DPN) (95/100%), adenosine triphosphate (ATP) and cytochrome *c* were obtained from L. Light and Co. Ltd. Triphosphopyridine nucleotide (TPN) was provided by Dr W. N. Aldridge and contained at least 70% of TPN (assayed with *iso*citric dehydrogenase) and no detectable DPN.

Glaxo–Wistar albino rats were killed by decapitation. Males were used unless otherwise stated.

Liver preparations. Liver slices about 0·5 mm. thick were cut and trimmed by hand to about 5 mm. square. They were washed twice in 0·9% (w/v) NaCl solution at room temp. before use.

For the preparation of homogenates, washed slices of liver were damp-dried on filter paper, chopped with scissors, weighed and homogenized in 2 ml. of ice-cold medium (isotonic sucrose or NaCl) in an ice-cold tube of the Potter-Elvehjem type. More liquid was then added to give a final volume of 4 ml./g. fresh weight of liver.

Cell-free suspensions were prepared by centrifuging the whole homogenate at 600 *g* for 5 min. at room temp., and withdrawing the supernatant. Chilled glassware was used throughout. Such suspensions had a cholinesterase activity, measured as described below, of about 5 μmoles of acetylcholine/ml./hr.

Further fractionation of homogenates in 0·25 M sucrose was carried out at 0° by the method of Schneider (1948). Cells and nuclei were removed by centrifuging at 600 *g* for 10 min., mitochondria by a further 10 min. at 8500 *g*, and microsomes by a further 60 min. at 18000 *g*. Each sediment was resuspended without washing in 0·25 M sucrose to the original volume of whole homogenate.

Boiled liver extract was made by dropping fresh chopped rat liver into boiling water (2 ml./g.) and boiling for 2 min.

The liver was homogenized and then centrifuged for 5 min. at 600 *g*. The supernatant liquid was used.

Preparation of rat-brain cholinesterase. A whole rat brain was washed free from blood with isotonic saline and homogenized in about 2 ml. of water. Further water was added to a final volume of 20 ml./g. fresh wt. of brain. This suspension was kept at room temp. for about 2 hr. and then centrifuged at 600 *g* for 10 min. The supernatant was withdrawn and stored at −5°. It maintained its cholinesterase activity for at least a month. The preparation hydrolysed acetyl-β-methylcholine readily, but butyrylcholine hardly at all. Its activity as measured by the method described below was about 20 μmoles of acetylcholine/ml. of preparation/hr.

Determination of cholinesterase. A manometric technique modified from that of Ammon (1933) was used. Each flask contained 2·0 ml. of Krebs–Ringer bicarbonate solution (see Umbreit, Burris & Stauffer, 1949), 0·5 ml. of the cholinesterase solution to be estimated, and 0·5 ml. of acetylcholine bromide (0·036 M) which was tipped from the side arm after the flasks had been gassed for 10 min. at 37° with $CO_2 + N_2$ (1:19). After a further 10 min., manometer readings were taken at 10–20 min. intervals for calculation of CO_2 output.

Measurement of direct anticholinesterase effect. The inhibitor in isotonic saline (0·25 ml.) was added at zero time to a Warburg flask containing 2·0 ml. of Krebs–Ringer bicarbonate and 0·25 ml. of cholinesterase preparation, with 0·5 ml. of acetylcholine bromide (0·036 M) in the side arm. The flask was transferred at once to a water bath at 37° and shaken continuously. During the last 10 min. of the inhibition period (usually 30 min.) $CO_2 + N_2$ (1:19) was passed through the flask, and the acetylcholine was finally tipped in to stop the inhibition reaction and measure the residual cholinesterase activity. Controls contained no inhibitor.

Conversion of dimefox by liver slices. In a typical experiment, each Warburg flask contained 0·9 ml. of rat-brain cholinesterase preparation, 0·9 ml. of double-strength Krebs–Ringer solution, and 0·2 ml. of 0·1 M phosphate buffer (Umbreit *et al.* 1949) containing dimefox at the required concentration, in the side arm. Six liver slices were added (total dry wt. about 7 mg.) and the dimefox was tipped in after a 5 min. equilibrating period at 37°. Controls were run containing (*a*) liver slices but no dimefox, and (*b*) dimefox but no liver slices, to detect direct inhibition by dimefox. At the end of the incubation, the media were decanted from the slices, and 2 × 0·5 ml. of each was used for duplicate cholinesterase determinations.

Conversion of dimefox by liver homogenates. Inhibition of endogenous acetylcholinesterase activity of whole homogenates of liver or cell-free suspensions was used as a measure of the inhibitor produced. Generally, each flask contained 1·6 ml. of suspension in 0·25 M sucrose, 0·2 ml. of 0·25 M sucrose containing the activator or inhibitor being studied and 0·2 ml. of 0·1 M phosphate buffer pH 7·4 containing dimefox. The flasks were shaken in air at room temp. (20 ± 2°) and at the end of the incubation period 2 × 0·5 ml. of the contents was taken for cholinesterase determination and gassed at once with $CO_2 + N_2$ (1:19). Blank determinations (omitting acetylcholine) showed that the evolution of CO_2 due to the anaerobic production of lactic acid by the liver suspension was negligible. The direct inhibition of cholinesterase by unchanged dimefox was determined after gassing with $N_2 + CO_2$ to prevent conversion occurring, or by using liver suspensions in water which contained cholinesterase but would not metabolize dimefox. In subsequent experiments, dimefox concentrations which had no direct effect on liver cholinesterase were used and dimefox was omitted from control flasks.

RESULTS

Direct inhibition of cholinesterase

The direct effects of dimefox on rat- and human-blood cholinesterase were determined (Table 1). Whereas rat-erythrocyte cholinesterase was inhibited 50 % by $2{\cdot}2 \times 10^{-3}$ M dimefox in 30 min. at 37°, tetraethyl pyrophosphate produced the same effect at a concentration of about 10^{-8} M. This was taken as an indication of the freedom of the dimefox

Table 1. *Direct effect of dimefox on rat- and human-blood cholinesterase* in vitro

Dimefox was incubated with rat erythrocytes or plasma diluted with 3 vol. of water, or human erythrocytes or plasma diluted with 59 vol. of water, for 30 min. at 37° before adding acetylcholine bromide and measuring residual cholinesterase activity (see Methods section).

Cholinesterase preparation	Dimefox (M) concn. producing No inhibition	Dimefox (M) concn. producing 50 % inhibition
Rat erythrocytes	10^{-4}	$2{\cdot}2 \times 10^{-3}$
Rat plasma	10^{-4}	$3{\cdot}3 \times 10^{-3}$
Human erythrocytes	10^{-3}	$1{\cdot}1 \times 10^{-1}$
Human plasma	10^{-3}	$1{\cdot}9 \times 10^{-2}$

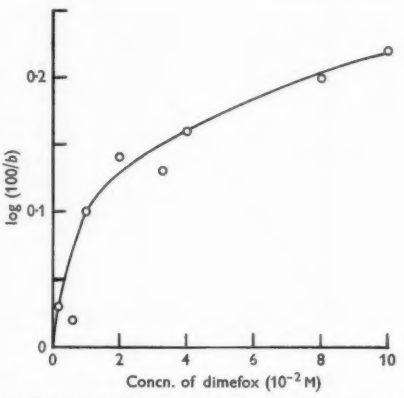


Fig. 1. Direct inhibition of rat-brain cholinesterase by dimefox. Dimefox was incubated with rat-brain cholinesterase for 15 min. at 37° before addition of acetylcholine bromide (see Methods section). *b* is the percentage of initial cholinesterase remaining.

sample from significant contamination by any direct anticholinesterase.

The nature of the direct inhibition of enzyme by high dimefox concentrations was examined briefly, although it probably has no practical significance *in vivo*. A straight line could not be obtained by plotting log (100/*b*) (where *b* = percentage of initial cholinesterase remaining) against dimefox concentration (Fig. 1), or against time with a fixed concentration of dimefox (see Aldridge, 1950). The rate of the inhibition reaction declined rapidly after about 10 min., possibly owing to the setting up of an equilibrium, since the inhibition of intact rat erythrocytes could be reversed by washing twice in three volumes of normal saline at room temperature.

Conversion of dimefox by liver slices

Incubation of dimefox (10^{-4}–10^{-1} M) with rat-liver slices for 1–2 hr., followed by application of the incubation medium to cholinesterase solutions (involving a 1/25 dilution) for 30 min., produced small and inconsistent inhibitions.

When the cholinesterase preparation was incubated with the slices and dimefox, more substantial and reproducible inhibitions were obtained. Dimefox (5×10^{-5} M) incubated in Krebs–Ringer phosphate (Umbreit *et al.* 1949) pH 7·4 at 37° in air for 15 min. with about 8 mg. (dry weight) of rat-liver slices and rat-brain cholinesterase caused about 50 % inhibition of cholinesterase. The dependence of the effects on incubation time and dimefox concentration is shown in Figs. 2 and 3. Log (100/*b*) was roughly proportional to dry weight of liver slices, although the consistency of results was not generally enhanced by correcting for small variations in liver weight between incubation flasks. This was presumed to be due to variations of slice thickness and, perhaps, of distribution of the enzyme system in them. The percentage inhibition was independent of the concentration of brain cholinesterase added to the system, and the effect on intact rat erythrocytes was not reversible by washing in normal saline at room temperature.

Stability of the metabolite. Since no difficulty has been reported by other workers in demonstrating an anticholinesterase in the medium after incubating schradan or parathion with liver slices, the stabilities of the three metabolites in Ringer phosphate, pH 7·4 (in which the slices had been incubated) were compared, as shown in Table 2.

Inhibition of conversion of dimefox by liver slices. The effects of various incubation conditions and inhibitors were investigated using rat-liver slices and brain cholinesterase. The presence or absence of 0·1 % (w/v) glucose in the medium was immaterial. Air was as effective as oxygen as gas phase provided the flasks were adequately shaken. Replacement of the air with $CO_2 + N_2$ (1:19) prevented all conversion, however. The effects of other inhibitors of

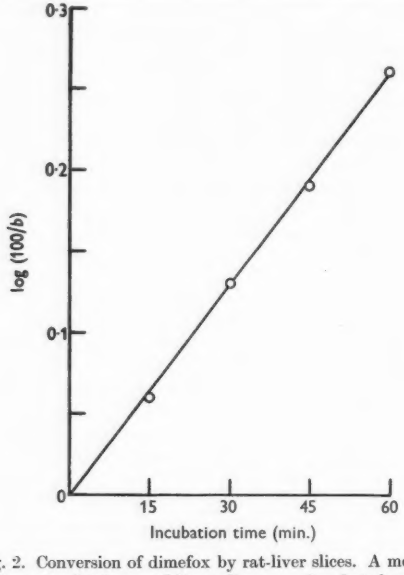


Fig. 2. Conversion of dimefox by rat-liver slices. A mean dry wt. of 6·1 mg. of liver slices was incubated with 5×10^{-5} M dimefox and rat-brain cholinesterase at 37° in a total volume of 2 ml. The gas phase was O_2 and the pH 7·4. Cholinesterase was determined in the medium at the end of the incubation. Controls contained slices but no dimefox.

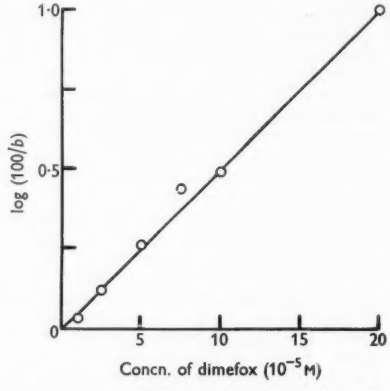


Fig. 3. Conversion of dimefox by rat-liver slices. A mean dry wt. of 8·1 mg. of liver slices was incubated with dimefox and rat-brain cholinesterase at 37° for 15 min. in O_2 at pH 7·4. The total volume of liquid was 2 ml. Cholinesterase was determined in the medium at the end of the incubation. Controls contained slices but no dimefox.

conversion are shown in Table 3, together with their effects on the overall oxygen uptake of the slices.

The addition of dimefox up to 0·1 M to liver slices with or without glucose in the medium did not increase the Q_{O_2} of the slices.

The concentration of cyanide in a flask with alkali in the centre well is uncertain. Therefore, the inhibitory effect was confirmed by using 10^{-4} M dimefox, incubated for 15 min. with liver slices, without KOH, and without gassing. Potassium cyanide (5×10^{-4} M) inhibited the conversion 38 and 45%, and 2×10^{-3} M-KCN 65 and 71% in two experiments.

Barbiturates disrupt oxidative phosphorylation *in vitro* at low concentrations (Johnson & Quastel, 1953; Brody & Bain, 1954), possibly inhibiting the oxidation of reduced DPN (Quastel, private communication). It was found, however, that the

Table 2. *Relative stabilities of liver-slice metabolites of dimefox, schradan and parathion*

Inhibitors were produced by incubating about 10 mg. dry wt. of rat-liver slices with the organophosphorus compound in 2 ml. of Krebs–Ringer phosphate for 15 min. at 37°. The medium was decanted and its direct inhibitory effect on rat-brain cholinesterase measured at the final equivalent concentration of original compound shown, and at the same time at half this concentration for subsequent determination of half-life. The last column shows the results of parallel experiments with simultaneous activation and inhibition.

	Concn. for production (M)	Equivalent concn. for detection of inhibitor (M)	Stability of inhibitor	Concn. to cause 50% inhibition of rat-brain cholinesterase, present simultaneously, in 15 min. (M)
Dimefox	10^{-2}	10^{-3}	None detectable 1 min. after removal of slices	5×10^{-5}
Schradan	5×10^{-4}	5×10^{-3}	Half-life at 20° 1·5 hr.	5×10^{-5}
Parathion	5×10^{-6}	10^{-7}	No loss of activity in 91 hr. at room temp.	10^{-6}

Table 3. *Inhibitors of dimefox conversion by rat-liver slices*

About 9 mg. (dry wt.) of male rat-liver slices was incubated with dimefox (2×10^{-5} M) and rat-brain cholinesterase for 1 hr. at 37° in Warburg flasks with 10% KOH in the centre wells, and gassed with O_2. Inhibitors were present initially except in the case of dinitrophenol, which was tipped from the side arm with the dimefox after 40 min. at 37°. O_2 uptake was measured over 45 min. during the incubation with dimefox and residual cholinesterase determined in the medium at the end of it. Inhibition of conversion is expressed as percentage decrease of log (100/b), where b is percentage of initial cholinesterase activity remaining. The mean control Q_{O_2} was 12·4, and the range 9·8–14·9 μl./mg. dry wt./hr.

Inhibitor	Concn. (M)	Inhibition of dimefox conversion (%)	Inhibition of Q_{O_2} (%)
$CO_2 + N_2$ (1:19)	—	100	—
KCN	2×10^{-3}	28	32
	4×10^{-3}	76	61
Nembutal	10^{-4}	60	0
	5×10^{-4}	79	42
Phenobarbital	10^{-4}	23	0
	5×10^{-4}	51	15
Thiopentone	10^{-4}	77	0
	5×10^{-4}	98	28
Chloretone	10^{-3}	20	0
	2×10^{-3}	53	18
2:4-Dinitrophenol	5×10^{-5}	5	(83% increase)
	5×10^{-4}	66	(16% increase)
SKF 525-A*	10^{-5}	49	0
	5×10^{-5}	100	0
Trimethylamine	10^{-3}	0	0
NN-Dimethylacetamide	10^{-3}	0	0
Dimethylnitrosamine	10^{-3}	0	0

* 2-(Diethylamino)ethyl diphenylpropylacetate hydrochloride.

addition of ATP (10^{-3} M) or DPN (10^{-3} M) did not reduce the effect of Nembutal [sodium 5-ethyl-5-(1-methylbutyl) barbiturate] on dimefox conversion. Subsequently it was noticed that the inhibitory effect of Nembutal (5×10^{-4} M) was greater at low concentrations of dimefox than at higher ones, e.g. 93 % inhibition of conversion of 2×10^{-5} M and 57 % of 10^{-4} M dimefox, indicating a competitive effect.

Measurement of liver efficiency. Units of anti-cholinesterase produced in a constant time with a constant dimefox concentration by a known weight of liver slices have been expressed as 100 log (100/*b*)/mg. dry wt. The results are shown in Table 4. Male rat liver was found to be about four times as efficient as female in converting dimefox. Guinea pigs were about twice and rabbits about five times as efficient as male rats. One slice of rabbit liver (less than 1 mg. dry wt.) was sufficient to cause 50 % inhibition of rat-brain cholinesterase in 15 min. with 5×10^{-5} M dimefox.

Table 4. *Relative efficiencies of slices of rat, guinea-pig and rabbit liver in converting dimefox*

Two different weights of slices from each animal were incubated for 15 min. at 37° with 5×10^{-5} M dimefox and rat-brain cholinesterase, before determination of residual cholinesterase activity in the medium.

	No. of animals	Liver efficiency* ± S.D.	Intra-peritoneal acute LD_{50} (mg./kg.)
Rat, female	3	1·6 ± 0·95	0·5
Rat, male	4	6·4 ± 1·8	0·5
Guinea pig, male	3	13 ± 2·1	2
Rabbit, male	2	31 ± 9·5	3

* Liver efficiency was calculated as 100 log (100/*b*)/mg. dry wt. of liver, where *b* is the percentage of initial cholinesterase activity remaining.

Liver homogenates

In accordance with reports on schradan and parathion (Metcalf & March, 1953; Casida *et al.* 1954; Davison, 1955; O'Brien & Spencer, 1955), 20 % homogenates of rat liver in 0·25 M sucrose or 0·154 M-NaCl showed no evidence of conversion of 10^{-4} M dimefox.

The addition of a boiled liver extract to an isotonic sucrose homogenate resulted in some conversion of dimefox, as indicated by inhibition of endogenous cholinesterase, and this was enhanced by the addition of 10^{-2} M nicotinamide and further by 10^{-2} M-$MgSO_4$.

When the liver was homogenized in isotonic sucrose or sodium chloride containing 10^{-2} M nicotinamide, significant conversion of added dimefox occurred. Centrifuging at 600 *g* for 5 min., to remove cell debris and probably most of the nuclei, did not result in loss of activity, although some of the cholinesterase was lost. Addition of nicotinamide about 10 min. after homogenizing had no effect, but if nicotinamide and DPN (10^{-3} M final concentration) were added together at this stage, conversion of dimefox again took place. TPN was equally effective in fortifying the homogenate in the presence of nicotinamide. The following experiment was carried out with the object of removing coenzymes and ATP from the homogenate and avoiding the conversion of DPN into TPN. A lightly centrifuged (5 min. at 600 *g*) liver homogenate (10 ml.) in 0·25 M sucrose containing 10^{-2} M-$MgSO_4$ was dialysed at 4° against two separate litres of isotonic sucrose–$MgSO_4$ solution for 7 hr. and a further 17 hr. respectively, with continuous stirring. It could still convert 10^{-3} M dimefox in the presence (but not in the absence) of added DPN and nicotinamide, but TPN could not now replace DPN.

Table 5. *Relative efficiencies of male rat-liver preparations in converting dimefox*

Liver preparations (1·6 ml.) were incubated with dimefox (10^{-4} M) in a total vol. of 2 ml. for 15 min. at 20° and pH 7·4 in air before measurement of residual endogenous cholinesterase activity. Slices were incubated in Krebs–Ringer phosphate and were homogenized and centrifuged for 5 min. at 600 *g* after incubation.

Liver preparation	Fortification	*b**	Equivalent wet wt. of liver (mg./2 ml.)	Efficiency†
Slices	None	46	120	28
Whole homogenate in 0·25 M sucrose 4 ml./g. wet wt.	None	90	360	1·3
Supernatant from sucrose homogenate after 5 min. at 600 *g*	None	93	450	0·7
	Boiled liver extract	78	350	3·1
	$MgSO_4$, 10^{-2} M	80	450	2·2
	Boiled liver extract + $MgSO_4$	31	350	15
	Boiled liver extract + $MgSO_4$ + nicotinamide 10^{-2} M	17	350	22
	Nicotinamide + DPN 10^{-3} M	70	450	3·4
	Nicotinamide + DPN + $MgSO_4$	33	450	11

* *b* is the percentage of initial cholinesterase remaining. † 10^4 log (100/*b*)/mg. wet wt. of whole liver/2 ml.

The addition of 10^{-3} M ATP or 10^{-4} M cytochrome *c* to a homogenate in sucrose–nicotinamide solution did not enhance its activity.

The efficiencies of various liver preparations in converting dimefox are shown in Table 5. Slices were the most efficient converters, but the supernatant from a homogenate fortified with $MgSO_4$, nicotinamide and boiled liver extract was almost equally active, and one with magnesium sulphate, nicotinamide and DPN was about half as active as slices.

Homogenates of washed liver slices were more active than those of unwashed chopped liver, possibly because of the removal of blood. Greater inhibition of liver cholinesterase was obtained at 20° than at 37°, owing, probably, to the greater stability of the converting system at the lower temperature.

Stability of suspensions. The activity of a sucrose–nicotinamide suspension declined rapidly at room temperature, falling to zero in about 1 hr. If the suspension was stored at 4°, its activity could be preserved for at least 3 hr. but had disappeared within 24 hr. The rate of decline of dimefox-converting power was about the same as that of ability to reduce methylene blue, and the Q_{O_2} was maintained steadily for at least 2 hr.

DPN (10^{-3} M) and nicotinamide (10^{-2} M) were added to a suspension in sucrose–$MgSO_4$ which had stood for varying times at room temperature. The dimefox-converting capacity of the fortified suspension decreased as its age increased, falling almost to zero in 2 hr. Cooper & Brodie (1954) reported that reduced TPN was needed by the liver to oxidize barbiturates. We added ethanol (10^{-1} M) with DPN and nicotinamide to a fresh suspension and to one that had stood at room temperature for 1 hr., in order to generate DPNH via the liver-alcohol dehydrogenase. No further enhancement of dimefox conversion occurred in either case. The labile factor is therefore probably not a DPNH-generating system.

Effect of pH. The effect of pH on the converting system was investigated by using Clark and Lubs phosphate and borate buffers (see Vogel, 1951). The activity increased steadily from pH 6 to 7·5, when a plateau was reached, no significant decline occurring up to pH 9 (Fig. 4). At the extremes of pH used, the cholinesterase itself was not affected, nor was the performance of the converting system influenced by changing from one buffer to the other at pH 8·0.

Effect of salts. Phosphate buffer could not be used in investigating the effect of salts on the system, because of the formation of precipitates. Therefore, Clark and Lubs borate buffer (pH 8·0) was used. $CaCl_2$ was most effective in enhancing the conversion of dimefox, followed by $MgSO_4$ and $BaCl_2$ (Table 6). NaCl, $MnCl_2$ and $CuSO_4$ had no effect, so it is probable that the activating effects observed were due to the metal ions rather than to the associated anions. In subsequent experiments, $MgSO_4$ was used when an activator was required, as it conveniently formed no precipitate with phosphate buffer.

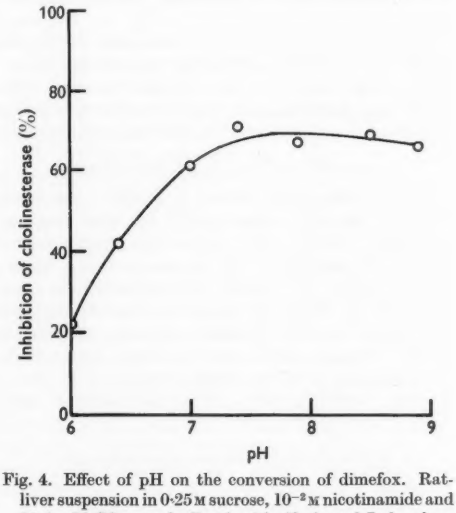


Fig. 4. Effect of pH on the conversion of dimefox. Rat-liver suspension in 0·25 M sucrose, 10^{-2} M nicotinamide and 10^{-2} M-$MgSO_4$ was buffered with Clark and Lubs phosphate and borate buffers (0·02 M). The suspension was incubated at 20° for 15 min. in air with 5×10^{-5} M dimefox, followed by determination of endogenous cholinesterase.

Table 6. *Effect of metal salts on dimefox conversion by rat-liver suspensions*

Male rat-liver homogenate in 0·25 M sucrose with 10^{-2} M nicotinamide (4 ml./g. wet wt.) was centrifuged for 5 min. at 600 *g*. Supernatant (1·6 ml.) was incubated for 15 min. at 20° in air with 5×10^{-5} M dimefox in borate buffer (0·02 M) pH 8·0 and salts as shown. Total vol., 2·0 ml. Cholinesterase activity was determined at the end of the incubation.

Salt	Concn. of activator (M)	Inhibition of liver cholinesterase (%)
None	—	12
$CaCl_2$	10^{-4}	10
$CaCl_2$	10^{-3}	47
$CaCl_2$	10^{-2}	55
$MgSO_4$	10^{-2}	36
$BaCl_2$	10^{-2}	26
$MnCl_2$	10^{-2}	3
$CuSO_4$	10^{-2}	7
NaCl	10^{-2}	9
KCl	10^{-2}	15

Centrifugal fractionation of liver homogenates. After a liver homogenate in 0·25 M sucrose had been fractionated into cells and nuclei, mitochondria, microsomes and supernatant, most of the cholinesterase was found in the first two fractions, and the enzyme activity was low. Therefore, rat-brain cholinesterase was added to each fraction and used as a control, the endogenous liver cholinesterase being negligible by comparison. The results are shown in Table 7.

It was found, in one experiment, that TPN was as effective as DPN in activating the (microsomes + supernatant) system, and, in another, that the addition of a boiled liver extract to the microsome fraction did not enhance its activity materially.

Conversion of dimefox by other tissues

No evidence of activation of dimefox has been obtained with rat kidney, brain (cerebral cortex), duodenum, ileum, colon, leg muscle, heart muscle, thyroid or adrenal. With muscle, gut and brain, the inhibition of endogenous cholinesterase was measured by using 10^{-4} M dimefox and incubating for up to 3 hr. With kidney, thyroid and adrenal slices or bisects, brain cholinesterase was added. Homogenates of whole brain and ileum in 0·25 M sucrose, fortified with DPN, nicotinamide and $MgSO_4$, were also inactive.

Effect of plasma on dimefox conversion

Dimefox activated by liver slices was found to have considerably less effect on rat-plasma cholinesterase than on erythrocyte or brain cholinesterase. Also, the cholinesterase of rat erythrocytes suspended in plasma was less inhibited than that of erythrocytes in Krebs–Ringer phosphate, the magnitude of the effect varying with the concentration of plasma in the suspending medium. A similar phenomenon was noted with human erythrocytes and plasma (Table 8).

The following possible explanations of this effect were tested: (*a*) That dimefox was rapidly destroyed by an enzyme in plasma. Pre-incubation of 10^{-3} M dimefox in plasma for 15 min. at 37° did not reduce the subsequent effect on brain cholinesterase with liver slices at a 1/10 dilution. (*b*) That the inhibited erythrocyte cholinesterase was reactivated in the presence of plasma, as has been noted to occur with the insecticide dimethyl dichlorovinyl phosphate (M. L. Fenwick, unpublished observation). Erythrocytes, after inhibition by activated dimefox, were incubated for 15 min. at 37° in plasma. No recovery of activity occurred. (*c*) That plasma contained some material that had a higher affinity for activated dimefox than had erythrocyte cholinesterase, and competed with it. Plasma was pre-incubated for 15 min. with 10^{-3} M dimefox and liver slices to saturate all possible phosphorylatable sites. It subsequently still had the same effect as untreated plasma in reducing the inhibition of erythrocytes suspended in it.

These three possibilities having been eliminated, it was concluded that some material, possibly an enzyme, in plasma rapidly destroyed the activated dimefox before it could attack the erythrocyte cholinesterase. A similar effect on the inhibition of erythrocyte cholinesterase was noticed when plasma was replaced by lightly centrifuged aqueous homogenates of washed liver and kidney slices. Extracts of leg muscle, heart muscle, ileum, spleen or brain were inactive in this respect.

In view of the discovery of the inactivating material in liver tissue, the possibility was considered that magnesium sulphate, which fortified liver homogenates, might function by inhibiting the

Table 7. *Location of the dimefox-converting system in rat-liver cells*

Each precipitate was made up to volume of original 20% (w/v) homogenate in 0·25 M sucrose–10^{-2} M-$MgSO_4$. Each flask contained 0·8 ml. of brain-cholinesterase preparation, 0·4 ml. of liver fraction, 0·2 ml. of phosphate buffer (0·1 M, pH 7·4); DPN 10^{-3} M, nicotinamide 10^{-2} M, $MgSO_4$ 10^{-2} M, sucrose 0·25 M, dimefox 10^{-3} M final concentrations in a volume of 2 ml. Flasks were incubated 15 min. in air at room temp., followed by determination of cholinesterase on 0·5 ml. of contents.

Liver fraction	Inhibition of rat-brain cholinesterase (%)
Cells + nuclei	0
Mitochondria	12
Microsomes	33
Supernatant	0
Mitochondria + microsomes + supernatant	61
Mitochondria + supernatant	10
Microsomes + supernatant	53

Table 8. *Effect of plasma on the inhibition of erythrocyte cholinesterase*

Washed erythrocytes were suspended in plasma, or plasma diluted with Ringer solution and incubated for 15 min. at 37° with dimefox, and about 7 mg. dry wt. of male rat-liver slices. The slices were removed and the erythrocytes separated by centrifuging, washed and diluted with water (1/4 for rat; 1/20 for human) for residual cholinesterase determination.

Dimefox concn. (M)	Plasma concn. (%)	Inhibition of erythrocyte cholinesterase (%)	
		Rat	Human
10^{-3}	0	85	—
10^{-3}	100	12	—
10^{-4}	0	46	41
10^{-4}	12·5	35	30
10^{-4}	25	8	19
10^{-4}	50	0	0
10^{-4}	100	0	0

breakdown of converted dimefox. However, the addition of 10^{-2} M-$MgSO_4$ had no effect on the inhibition of erythrocyte cholinesterase, in the presence of a liver extract, with dimefox and liver slices.

The plasma inactivator was similarly unaffected by 10^{-3} M cupric or mercuric chlorides, in contrast to DFP-ase (Mounter & Chanutin, 1953) which hydrolyses di*iso*propyl phosphorofluoridate.

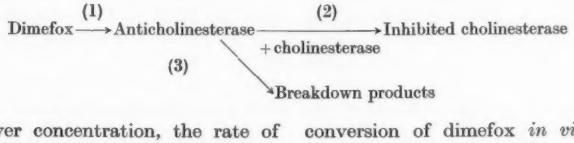
Conversion of dimefox by cockroach gut

Metcalf & March (1953) showed that the gut of the cockroach (*Periplaneta americana*) will convert parathion into an active anticholinesterase *in vitro*, and O'Brien & Spencer (1953, 1955) used cockroach gut to activate schradan. In both cases the homogenized tissue, variously fortified, was inactive.

Our experiments with dimefox are in accordance with these findings. The intact whole gut was active, causing inhibition of rat-brain cholinesterase when incubated with dimefox. But homogenates of the gut in 0·25 M sucrose, fortified with DPN (10^{-3} M), nicotinamide (10^{-2} M) and magnesium sulphate (10^{-2} M) effected no conversion of dimefox (10^{-4} M) in 15 min. at room temperature, when the endogenous acetylcholinesterase activity of the gut was used as an indicator of active inhibitor.

DISCUSSION

Dimefox, like schradan and parathion, has been shown to be converted into a powerful anticholinesterase in liver tissue. The results of our experiments with dimefox and rat-liver slices in the presence of rat-brain cholinesterase suggest that dimefox is metabolized by a relatively slow rate-limiting reaction (1) catalysed aerobically by a liver enzyme system; the product, a strong anticholinesterase, undergoes either an irreversible bimolecular reaction with cholinesterase (2) resulting in an inactive product or products, or a rapid spontaneous or (particularly *in vivo*) enzymic or chemical breakdown (3).



With a fixed liver concentration, the rate of reaction (1) is proportional to dimefox concentration, and does not decline appreciably within an hour under the conditions studied. The active anticholinesterase produced is present in small amount, as it is rapidly broken down by reaction (3), and its concentration is proportional to that of dimefox. If concentrations of dimefox, active inhibitor, and cholinesterase at time t are denoted by $[D]$, $[A]$ and $[C]$ respectively, and the percentage of initial cholinesterase activity remaining by b,

$$-\mathrm{d}[C]/\mathrm{d}t = k'[C]\,[A] = k[C]\,[D],$$

since $[A] \propto [D]$ (k', k are constants).

Integrating, $\ln(100/b) = k[D]t$. This relationship has been shown to be valid (Figs. 2 and 3). Similarly, since the rate of reaction (1) is proportional to concentration of liver enzyme, $\ln(100/b)$ is proportional to concentration of liver enzyme if $[D]$ and t are constant.

The quantity b is independent of $[C]$, other conditions remaining constant [unless the cholinesterase preparation also contains some factor that affects the rate of breakdown of A, reaction (3)].

The technique used to study this system, involving all three reactions, has given satisfactory consistency of results, and can be used as an assay method for either the liver-enzyme system, or low concentrations of dimefox.

Our results do not agree with those of Okinaka *et al.* (1954). The dimefox used by these workers had a high anticholinesterase activity, which was reduced by incubating with liver slices. A possible explanation is that it was contaminated with a powerful inhibitor which was destroyed by liver tissue, whereas the dimefox metabolite, being very unstable, remained undetected.

Brodie (1956) has reviewed work on a (liver microsomes + supernatant) system which accomplishes the oxidative metabolism of various drugs, including barbiturates, and Davison (1955) has compared this system with the one that oxidizes schradan and parathion.

Male rats are more resistant than female rats to anaesthetizing doses of barbiturates (Holck, Kanan, Mills & Smith, 1937), and we have noticed that male rat liver is a more efficient converter of dimefox, *in vitro*, than is female. SKF 525-A [2-(diethylamino)ethyl diphenylpropylacetate hydrochloride] inhibits both barbiturate metabolism and dimefox (and schradan and parathion) conversion. Furthermore, low concentrations of barbiturates inhibit the conversion of dimefox *in vitro*, and this effect cannot be reduced by addition of ATP or DPN. The barbiturates do in fact appear to compete with dimefox for the use of some part of the oxidizing system, which must have a very broad specificity. The reasons for the inhibitory effect of chloretone and dinitrophenol are not at present clear, although possibly they, too, undergo oxidation by the same system. Two compounds with dimethylamino

groups, *NN*-dimethylacetamide and dimethylnitrosamine did not inhibit dimefox conversion, however.

A difference between the dimefox-converting system and Brodie's drug-metabolism system is that DPN is an effective cofactor in the former, whereas DPN could not replace TPN in barbiturate metabolism.

The system that metabolizes dimefox is very unstable in liver homogenates, partly, but not wholly, owing to the rapid loss of DPN. Brodie and his colleagues suggest that a peroxide-like oxidizing agent is produced during the oxidation of TPNH. We considered the possibility that liver homogenates 'ran down', even in the presence of DPN, because of lack of DPNH. We found no increased dimefox conversion, however, on addition of ethanol to accelerate the production of DPNH via the alcohol dehydrogenase presumed to be present. On the other hand, cytochrome *c* also had no activating effect, although the system was inhibited by cyanide. The mechanism is evidently far from clear.

A few experiments with cockroach gut showed that, although the intact tissue will convert dimefox *in vitro*, homogenates fortified with nicotinamide, DPN and magnesium sulphate will not do so. It appears that there is a difference between the activating systems in rat liver and in cockroach gut—either a fundamental difference of mechanism or a difference in stability on homogenizing.

A close and not surprising similarity has been observed between the behaviours of dimefox and the related compound schradan. It may be surmised that, by analogy with the metabolism of schradan (Heath, Lane & Llewellyn, 1952; Casida *et al.* 1954; Heath, Lane & Park, 1955), the anticholinesterase derived from dimefox is also either an amine oxide or a hydroxymethylamide. The most obvious difference lies in the relative instability of the dimefox liver metabolite *in vitro*. While 10^{-2} M dimefox, activated by liver slices, had no detectable inhibitory activity when transferred within 1 min. to cholinesterase at 1/10 concentration, 5×10^{-5} M dimefox, incubated with cholinesterase and the same weight of liver slices, produced 50 % inhibition in 15 min. The presence of an inactivator in liver no doubt reduces the survival time of the active material. (We have obtained a strong anticholinesterase in small yield, by oxidizing dimefox with hydrogen peroxide and extracting with chloroform. This was somewhat more stable and had a half-life of about 8 min. at pH 7·4 at 37°.) The additional presence of a similar material in plasma must further reduce the half-life of activated dimefox *in vivo*, as shown by the strong protective effect of plasma on erythrocyte cholinesterase *in vitro*.

Although concentrations and stabilities are very difficult to estimate with any accuracy in such a system under living conditions, some suspicion is cast on the ability of the liver metabolite of dimefox to reach a peripheral site in the blood in sufficient concentration to produce lethal effects. With this in mind we have tried to demonstrate conversion in various other organs and tissues, but without success. The effect may, of course, be more localized and not demonstrable by our macro-techniques. It can be seen from Table 4 that an efficient liver conversion cannot be correlated with a low LD_{50} in the rat, rabbit and guinea pig. On the other hand, there is indirect evidence that the liver is the critical site of production of an unstable toxic substance; symptoms of poisoning are slow to appear, often not starting for 2 or 3 hr. after an intraperitoneal LD_{50} dose, and death may only occur after 2 or 3 days; brain cholinesterase is inhibited to a much lesser extent than is erythrocyte cholinesterase after dimefox injection; previous treatment of rats with carbon tetrachloride reduces the rate of depression of plasma cholinesterase after injection of dimefox, and also delays the onset of external symptoms of poisoning (M. L. Fenwick, unpublished observation).

Work is continuing on these aspects of dimefox toxicology.

SUMMARY

1. Dimefox is a weak anticholinesterase, and its direct effect on rat-erythrocyte cholinesterase may be reversed by washing the intact cells.

2. Dimefox is converted into a powerful and unstable anticholinesterase in liver tissue. A technique for investigating the system *in vitro* has been described.

3. The stability of the metabolite is further decreased by the presence in liver, blood and kidney of a substance which inactivates it.

4. The converting system is present in the microsomes and soluble material of the liver cells, and bears certain resemblances to the system which metabolizes barbiturates and other drugs. It is probably the same as that which activates schradan.

5. The conversion in liver suspensions is accelerated by DPN and nicotinamide, and by Ca^{2+} or Mg^{2+} ions. It is inhibited in liver slices by lack of oxygen, by barbiturates, SKF 525-A [2-(diethylamino)ethyl diphenylpropylacetate hydrochloride], 2:4-dinitrophenol and potassium cyanide.

6. No site of conversion of dimefox in the rat other than liver could be demonstrated *in vitro*.

7. Conversion of dimefox also takes place in whole cockroach gut *in vitro*, but homogenates fortified with DPN, nicotinamide and magnesium were inactive.

We wish to thank Dr E. F. Edson for his continual interest and encouragement, and Dr W. N. Aldridge for advice, for a sample of TPN, and for laboratory facilities during a part

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The Phosphatase and Metaphosphatase Activities of Pea Extracts

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Metaphosphates and inorganic polyphosphates are widespread in living organisms (see reviews by Ingelman, 1950; Schmidt, 1951; Winkler, 1953). Apart from the ubiquitous formation of pyrophosphate by various enzyme reactions, large amounts of these polyphosphates have been detected in moulds, bacteria, algae, the seeds of cotton plants and some insect tissues, although not so far in the higher animals or the leaves of higher plants. Enzymes which degrade the inorganic polyphosphates, with or without the liberation of orthophosphate, have also been detected in extracts of a wide range of organisms (see Ingelman, 1950; Schmidt, 1951). There have, however, been few studies on the purification and properties of these enzymes.

In the course of previous work on the purification of plant ribonuclease (Holden & Pirie, 1955*a*; Pierpoint, 1956), it was observed that pea-leaf extracts would liberate orthophosphate from commercial 'hexameta-phosphate'. In view of the increasing interest in the occurrence and metabolism of poly- and meta-phosphates, this metaphosphatase has been characterized further. In particular, some attention has been paid to its specificity, since very few of the metaphosphatases described have been tested against more than one or two inorganic polyphosphates.

Evidence is presented here that the metaphosphatase activity of the pea extracts is associated with an unspecific phosphoesterase, which liberates orthophosphate from trimetaphosphate and tripolyphosphate as well as a number of organic phosphates. During the purification of this enzyme by ion-exchange chromatography, it was resolved into two distinct components with similar but not identical properties.

EXPERIMENTAL

Enzyme preparations. Extracts of pea seedlings that had been grown in a glasshouse for two or three weeks were fractionated by the method of Holden & Pirie (1955*a*). The fractions are referred to by the letters that these workers used. Many of the experiments were performed with fraction E, since it contained most phosphatase activity on a protein-nitrogen basis. It was prepared by precipitation with ammonium sulphate between 50 and 85% saturation, from a citrate extract of pea seedlings that had been clarified

at pH 4·5. The precipitate was dissolved in citrate buffer at pH 5, dialysed and then centrifuged.

Enzyme estimations. The various phosphatase activities were measured in terms of the inorganic orthophosphate liberated from the substrates.

Glycerophosphatase activity was measured by incubating samples of the enzyme for 1 hr. at 37° in a solution containing β-glycerophosphate at a concentration of 0·4 mg. of P/ml., and 25 mM citrate buffer (pH 5) in a total volume of 4 ml. Samples were taken before and after incubation and usually pipetted directly into the sulphuric acid used in the phosphate assay. However, when the activity of unfractionated sap was being measured, or shorter periods of incubation were used, the reaction was stopped and protein removed by the addition of 2 ml. of 15% trichloroacetic acid. Samples for phosphate assay were then taken after the precipitate had been removed by centrifuging for 10 min. at 3000 rev./min.

Phosphatase activity against the polyphosphates adenosine diphosphate (ADP) and adenosine triphosphate (ATP) was measured in the same way with the substrates present at a concentration of 0·4 mg. of P/ml. The final pH was 6 instead of 5, since this was nearer the pH optimum for these substrates with fraction E. In more purified enzyme preparations the pH optimum for glycerophosphate, as well as these other substances, was near 6, and some of the glycerophosphatase measurements involving very small amounts of enzyme were done at this pH. These measurements are indicated in the appropriate places.

Phosphodiesterase activity was looked for by the liberation of phenol from diphenyl phosphate. The enzyme was incubated for 1 hr. at 37° in a solution containing diphenyl phosphate at a concentration of 0·4 mg. of P/ml. and citrate buffer 50 mM, in a total volume of 4 ml. at pH 5. Samples were taken before and after incubation and the phenol in them estimated with the Folin–Ciocalteu (1927) reagent.

The unit of phosphatase activity was defined as the activity that would liberate 31 mg. of P as orthophosphate/l. of solution, when incubated for 1 hr. under the conditions described. Since the liberation of phosphate did not vary in a linear manner with amount of enzyme added (Fig. 1), the assay was performed with various amounts of enzyme, and the amount containing one unit of activity measured graphically.

Chemical estimations. Orthophosphate was estimated as described by Holden & Pirie (1955*b*). The samples were pipetted into 5 ml. of 2N sulphuric acid; 1 ml. of 7·5% (w/v) ammonium molybdate was added, and water to 9 ml. The solution was shaken well before and during the addition of 1 ml. of freshly prepared stannous chloride (0·2%), and after a few minutes the colour was compared with a suitable standard on a visual colorimeter. Total phosphate was estimated in a similar way after digestion with a mixture of sulphuric and perchloric acids (Holden & Pirie, 1955*b*).

Nitrogen was determined by a micro-Kjeldahl method, and the ammonia measured either by titration with hydrochloric acid or by nesslerization. To determine the amount of protein nitrogen in enzyme solutions, an equal volume of 10% (w/v) trichloroacetic acid was added, and the precipitate centrifuged down at 5000 *g*, washed with 5% (w/v) trichloroacetic acid, and then dissolved in a small volume of NaOH solution before the Kjeldahl digestion.

Protein was also estimated in terms of its ultraviolet absorption at 280 mμ. in the 1 cm. cells of a Unicam SP. 500 spectrophotometer.

Chemicals. Tripolyphosphate ($Na_5P_3O_{10},6H_2O$) and the cyclic metaphosphates, trimetaphosphate [$(NaPO_3)_3,6H_2O$] and tetrametaphosphate [$(NaPO_3)_4,10H_2O$] were gifts from Albright and Wilson Ltd. The trimetaphosphate and tripolyphosphate contained 6 and 0·5% respectively of a phosphate that reacted as orthophosphate in the phosphate assay. The number of acid groups that could be titrated between pH 5 and 9 was consistent with these amounts of impurity, and paper chromatography in a *tert.*-butyl alcohol system (Crowther, 1954) indicated that the contaminant in the tripolyphosphate was orthophosphate, whereas that in the trimetaphosphate was probably pyrophosphate. No impurity could be detected in the tetrapolyphosphate.

The three phosphates, unlike the small amounts of contaminants, did not react in the phosphate assay, nor were they hydrolysed under the conditions of the assay.

Calcium phosphate was a three-year-old specimen that had been prepared by the method of Singer & Kearney (1950).

All the other chemicals were obtained commercially and were AnalaR as far as possible. The metaphosphoric acid (Hopkins and Williams Ltd.) contained 4–5% of orthophosphate.

Chromatographic methods. The procedure used for the chromatography of the enzymes was a pH–gradient elution method in which the sulphonated-polystyrene cation-exchange resin Dowex 50 (200–400 mesh, ×12) was used. The Dowex was converted into its sodium salt as described by Moore & Stein (1951) and packed into a glass column approximately 2 cm. × 35 cm. After each run it was regenerated by washing with 2N sodium hydroxide solution.

The eluting gradient was produced by a system essentially the same as that described by Boman (1955), and was almost linear over the effective range. The eluting buffer was 0·2M citrate (pH 6·7), which siphoned over from a 500 ml. conical flask into an open mixing vessel containing the

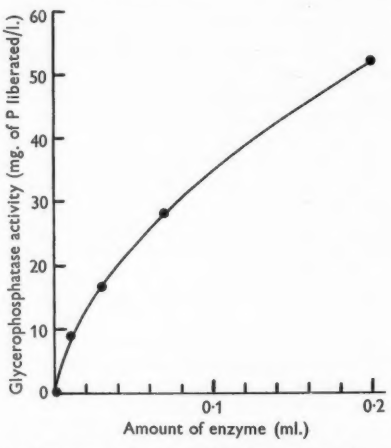

Fig. 1. Variation of glycerophosphatase activity with amount of enzyme. The enzyme used was a preparation of fraction E containing 0·16 mg. of protein nitrogen/ml. Enzyme activity was measured as described in the text.

0·2 M starting citrate buffer (pH 4·5). This mixing vessel was simply a piece of glass tubing of internal diameter 4 cm., with a rubber stopper as bottom. It is connected through this stopper to the flask of eluting buffer and the top of the column. The mixing of buffers in this vessel is promoted by a bubbler attached through a gas-washing bottle to an air pump.

At the beginning of each experiment the resin was washed well with 0·2 M citrate (pH 4·5) and then the enzyme solution, fraction E, containing 1000–2000 units of glycerophosphatase in 0·1 M citrate (pH 4·5) was added and allowed to sink into the resin. The column was then washed for 2–3 hr. with more buffer at pH 4·5. Elution was started by adjusting the level of citrate (pH 4·5) in the mixing vessel to that of the citrate (pH 6·7) in the conical flask, and opening the connexion between the two vessels.

Elution was continued for 24 hr. in the cold room at 2–4°. During this time the average flow rate was 22 ml./hr. Fractions of the eluate (10·1 ± 1·5 ml.) were collected in an automatic fraction collector of the second type described by James, Martin & Randall (1951).

All pH measurements were made with a glass electrode at room temperature.

RESULTS

Preliminary observations suggested that in a large number of pea extracts the ratio of glycerophosphatase to metaphosphatase was constant within the limits of the accuracy of the estimations. Table 1 shows some of these results obtained with fractions

Table 1. *Ratio of glycerophosphatase to metaphosphatase in pea-leaf extracts*

The fractions were obtained by the method of Holden & Pirie (1955*a*), and the enzyme activities measured as described in the Experimental section.

Expt.	Fraction	Further treatment	Glycero-phosphatase (units/ml.)	Meta-phosphatase (units/ml.)	Glycerophosphatase / Metaphosphatase
1	B	—	54	46	1·17
	E	—	94	92	1·02
	F	—	44	35·5	1·24
	J	—	42	34·4	1·26
	F	Ppt. at 0–50% saturation with $(NH_4)_2SO_4$	29	21	1·38
2	E	—	15·6	18·8	0·83
3	E	—	18·8	16·3	1·16
4	E	—	12·5	13·4	0·93
5	E	—	19·2	22·0	0·87
	E	Heated at 37° for 180 min.	18·3	18·8	0·97
	E	Heated at 56° for 45 min.	4·1	4·7	0·88

Table 2. *Phosphatase activities in fractions of pea extracts*

Solution E was fractionated either with cold ethanol (−10°), or, after its pH had been adjusted to 7, with ammonium sulphate. The precipitates were collected by centrifuging in the cold, dissolved in 0·1 M citrate buffer (pH 5), and dialysed overnight against water before being assayed for enzymic activity.

		Glycerophosphatase		Metaphosphatase		
Expt.	Fraction	(units/mg. of protein N)	Recovery (%)	(units/mg. of protein N)	Recovery (%)	Glycerophosphatase / Metaphosphatase
1	E	81	100	91	100	0·89
	Precipitate obtained on addition of 0–0·55 vol. of ethanol	51	11·7	58	11·4	0·88
	Precipitate obtained on addition of 0·55–1·66 vol. of ethanol	159	40	152	35	1·05
	Supernatants after addition of 3·0 vol. of ethanol	50	7·1	45	6	1·11
2	E	156	100	100	100	1·56
	Ppt. at 0–50% saturation with $(NH_4)_2SO_4$	116	6·3	120	10·2	0·97
	Ppt. at 50–60% saturation with $(NH_4)_2SO_4$	127	13	132	21	0·97
	Ppt. at 60–70% saturation with $(NH_4)_2SO_4$	200	19·2	152	23	1·30
	Ppt. at 70–80% saturation with $(NH_4)_2SO_4$	435	15	150	7·1	2·90

Table 3. *Adsorption of phosphatase and metaphosphatase on calcium phosphate gel*

Various amounts of phosphate gel were added to 4 ml. samples of fraction E containing 67 units of glycerophosphatase, and water to 6·5 ml. After standing at 0° for 10 min. the gel was removed by centrifuging, and enzyme activity, protein and dry-weight contents were measured in the supernatant solutions. Protein was estimated from the optical density at 280 and 260 mμ. (Warburg & Christian, 1941).

Phosphate gel added (mg. dry wt.)	Meta-phosphatase remaining (%)	Glycero-phosphatase remaining (%)	Dry wt. remaining (%)	Protein remaining (%)
0	100	100	100	100
1·77	88	91	89	89
5·3	52	51	—	73
11·6	13	19	68	61
21	1·6	3·0	61	51
45	<1	<1	58	45

Table 4. *Adsorption of phosphatase and metaphosphatase on Dowex 50*

Samples of solution E containing 1·8 units of glycerophosphatase in 80 mM citrate (pH 4·5), total volume 3·6 ml., were agitated for 15 min. at 0° with various amounts of the sodium salt of Dowex 50. The resin was removed by centrifuging and the enzymes were estimated in the supernatant solutions.

Amount of Dowex added (mg. dry wt.)	Meta-phosphatase remaining (%)	Glycero-phosphatase remaining (%)
0	100	100
40	60	55
120	5	0
200	0	0

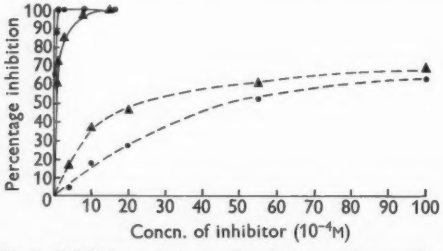

Fig. 2. Inhibition of glycerophosphatase and metaphosphatase activities. Samples of fraction E (0·05 ml.) were incubated at 37° with glycerophosphate (●) or metaphosphate (▲) at concentrations of 0·4 mg. of P/ml.; citrate, 50 mM (pH 6); sodium fluoride (broken lines) or ammonium molybdate; water to 4 ml. Samples were taken for orthophosphate estimations after 1 hr.

of the extracts prepared by the method of Holden & Pirie (1955*a*). Similar ratios were observed with solutions obtained during the chromatography of leaf ribonuclease (Pierpoint, 1956). Fractionation of E with ethanol did not materially affect this ratio (Table 2). Nor did ammonium sulphate fractionation normally, although in some experiments, as in the one illustrated, there was a twofold increase in the proportion of glycerophosphatase activity in the small fraction precipitated between 70 and 80 % saturation. The erratic nature of this effect makes it difficult to interpret. Total enzyme recoveries were low (50–60 %) in both fractionations (Table 2).

Phosphatase and metaphosphatase activities disappeared in similar manner when fraction E was allowed to stand for 10 min. in the presence of calcium phosphate gel. Both had completely disappeared from solution before 55 % of the total protein, measured spectrophotometrically, had been adsorbed (Table 3). Much smaller amounts of both enzyme activities were also adsorbed from citrate buffer (pH 4·5) on the sodium salt of Dowex 50 (Table 4).

The behaviour of the enzyme solution in the presence of both glycerophosphate and metaphosphate is consistent with the view that both activities belong to the same enzyme. Thus in the presence of optimum amounts of both substrates, at either pH 5 or 6, the amount of orthophosphate liberated and its rate of liberation are approximately equal to those from the most active substrate alone. Also consistent with this view is the effect of molybdate and fluoride on these two activities. Both are affected to approximately the same degree by these substances at pH 6 (Fig. 2). Moreover at pH 5 the effect of fluoride on both activities was slightly greater, and that of molybdate slightly less, than at pH 6. In these experiments, unlike those with purified preparations of phosphatase from human prostate (Reiner, Tsuboi & Hudson, 1955), there was no reversal of the fluoride inhibition at higher concentrations of fluoride.

London & Hudson (1955) have shown that the heat-denaturation properties of phosphatases are specific and can be used in demonstrating the association of different enzyme activities with the same protein. Consequently a few observations have been made on the inactivation by heat of

glycerophosphatase and metaphosphatase under different conditions of pH and temperature. In these experiments, suitable amounts of enzyme were incubated in the required conditions, and samples taken at various times. Enzyme activities in these samples were measured using an incubation time of 15 min., all the other conditions being the same as in the usual estimations. At pH 6 and 51·8°, both activities were destroyed in a manner that approximated to first-order kinetics. The heat-denaturation constants, expressed in hr.$^{-1}$, were 2·5 for glycerophosphatase and 2·1 for metaphosphatase. At pH 5 and 44·5° no loss in either enzyme could be detected in periods up to 100 min. In view of the great differences that were observed between different phosphomonoesterases by London & Hudson (1955), these results are compatible with the idea that the two enzyme activities are associated with the same protein.

Experiments already described indicate that the material responsible for both enzyme activities could be absorbed from buffer at pH 4·5 on the sodium form of Dowex 50. Other experiments indicated that it could be eluted at higher pH values. Attempts were therefore made to chromatograph the enzymes on a Dowex column, with buffer of gradually increasing pH as eluting agent. Under these conditions the enzyme was eluted from the column as two distinct components. Fig. 3 illustrates a typical result. In this experiment, 10 ml. of fraction E, containing 1000 units of glycerophosphatase, and a total of 1·4 mg. of protein nitrogen was added to the column in 0·1 M citrate (pH 4·5). In their distribution in the fractions of eluate both enzyme activities follow the same pattern. There is a small peak of activity, hereafter referred to as peak *A*, which appears when the eluate has reached a pH of 5·0–5·1, and a second larger peak, peak *B*, eluted at a pH of 5·6–5·7. Three different preparations of E have been examined and all show these two components. The proportions of the two components in different preparations, estimated as the areas under the curves, vary somewhat, but the pH values of the eluate at which they first appear seem constant. In nine experiments performed on the three samples of solution E, the averages and standard deviations of these pH values were $5{\cdot}0 \pm 0{\cdot}24$ and $5{\cdot}7 \pm 0{\cdot}18$ respectively.

The way in which metaphosphatase and glycerophosphatase follow the same elution pattern strongly indicates that they are both associated with the same protein. As described in the legend of

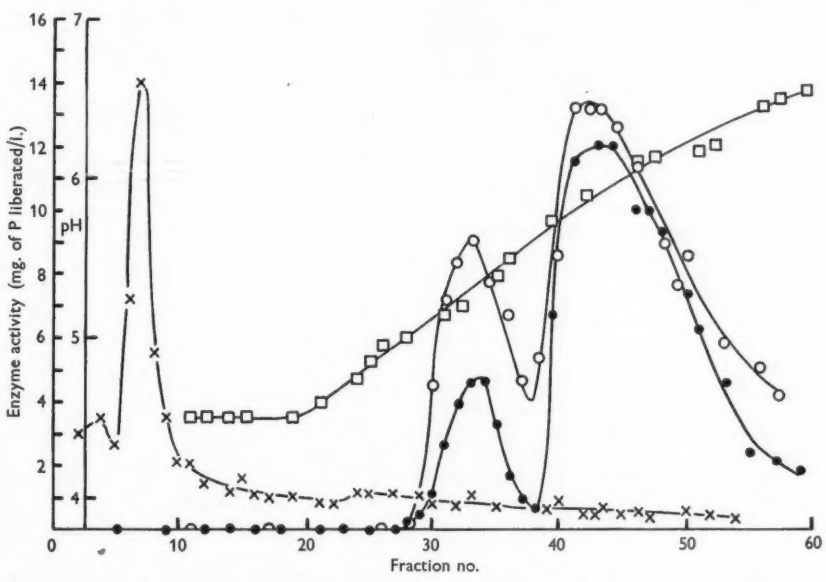

Fig. 3. Chromatography of pea phosphatase. A portion (10 ml.) of fraction E (1000 units of glycerophosphate, 1·4 mg. of protein) was chromatographed as described in the text. The pH values (□), optical density at 280 mμ.(×), glycerophosphatase (●) and metaphosphatase (○) activities were determined in each fraction. Enzyme activities were determined on 0·2 ml. samples by the method described, except that the concentration of the buffers was increased to 60 mM. The ordinate with optical density units is not shown, but the maximum value on the optical density curve is 1·4.

Fig. 3 the enzyme activities are estimated in single samples (0·2 ml.) of the fractions. Since the activity of the enzymes does not vary in a linear manner with the amount of enzyme present (Fig. 1), this estimation is not a strictly quantitative measure of the enzyme in the fractions. Consequently both enzyme activities were measured in the usual quantitative manner in eight selected fractions. If the fractions contained any activity at all, then the ratio of glycerophosphatase to metaphosphatase was constant, being independent of the peak of activity to which they belonged and of their position on that peak.

Further observations on the chromatography of the enzyme

It is evident from the curve of optical density at 280 mμ. in Fig. 3 that both components represent a considerable enrichment of enzyme activity. The bulk of the material in fraction E which absorbs at this wavelength is not adsorbed on the Dowex, but is washed straight through the column. It is not possible to measure accurately the amount of protein associated with the two components in terms of absorption at this wavelength, as the citrate buffers always had a small absorption, and there was the possibility of interference from resin material of low molecular weight (Boman, 1954). Estimations of protein N, however, give values for the specific activity of pooled *A* and *B* of 1440 and 1250 units of glycerophosphatase/mg. of protein N. These represent enrichments of the order of 7–10 times over the original fraction E. Because of the small amounts of enzyme used in these chromatographic runs, it has not proved possible to estimate the nitrogen associated with each enzyme component. Consequently it is not known if they have similar specific activities. The total amount of enzyme recovered was measured in a number of experiments and found to be between 50 and 60 % of that added to the column.

Although the formation of the two enzymic components could have been brought about by the chromatographic procedure itself, a number of experiments argue against this. Thus it has been possible to rechromatograph the components separately. Figs. 4 and 5 show the results of two experiments in which the fractions of eluate containing either component *A* or *B* were pooled and rechromatographed. Both experiments were performed on a sample of solution E containing the two components in a similar ratio to the one used in Fig. 3. The separated components behaved in a homogeneous manner and were eluted at the same pH as in the initial chromatograms. They were contaminated with 5 % or less of each other.

Another possible cause of the apparent inhomogeneity of an enzyme is the presence of traces of metal ions in the enzyme preparations (see e.g. Hirs, Moore & Stein, 1953). This is unlikely here because the incorporation of 8-hydroxyquinoline (2 mM) and disodium ethylenediaminetetraacetic acid (mM) into both the buffers used in the chromatography did not affect the elution pattern of the enzyme. These compounds had no effect on the estimation of the enzyme.

The relative amounts of the two components in a sample of E varied with the age of E. As a preparation aged the relative amount of component *A* in the total recovered enzyme increased. Fig. 6 illustrates this phenomenon and shows that it is due not to an increase in the amount of *A* present but rather to a decrease in the amount of *B*. Judged by the areas under the curve the proportion of *A* in this preparation increased from about 15 to 40 % during the

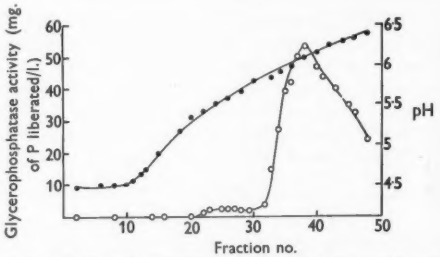

Fig. 4. Isolation of component *B*. The fractions containing *B*, derived from an experiment similar to that of Fig. 3, were pooled and chromatographed after adjustment of their pH to 4·5. Samples of the eluate were taken for pH (●) and enzyme (○) estimation. Glycerophosphatase was measured on 0·4 ml. samples by the method described, except that the buffer used was 100 mM citrate (pH 6), and that the incubation period was 2 hr.

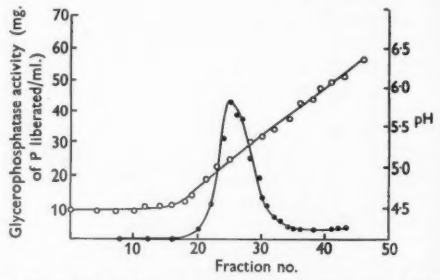

Fig. 5. Isolation of component *A*. The fractions containing *A*, derived from an experiment similar to that of Fig. 3, were pooled and chromatographed after adjustment of their pH to 4·5. The pH (○) and glycerophosphatase content (●) were measured on samples of the eluate. Glycerophosphatase was measured on 0·4 ml. samples by the method described, except that the buffer used was 100 mM citrate (pH 6), and the incubation time was 2 hr.

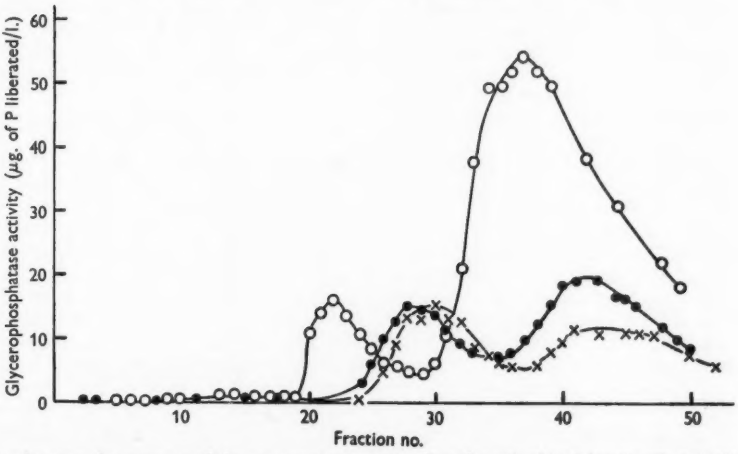

Fig. 6. Effect of age on the amounts of the enzyme components. Samples (10 ml.) of fraction E, initially containing 168 units of glycerophosphatase/ml., were chromatographed 1 day (○), 49 days (●), and 53 days (×) after preparation. Between the experiments the enzyme solution was stored at about 2°, and by the 49th day had lost 66% of its activity. Glycerophosphatase was estimated on samples (0·2 ml.) of the eluate as previously described, except that the buffer was 56 mM citrate (pH 6), and the incubation period was 2 hr. The peaks from the three different chromatographic runs do not coincide because of differences in the shape of the pH gradients in the three runs.

Table 5. *Heat stability of the two components of pea phosphatase*

Solutions of *A* and *B*, containing 2·8 and 6·7 units of phosphatase respectively, were adjusted to the required pH with molar citrate or citric acid, and incubated at the temperatures stated below. Samples were taken for enzyme estimations at appropriate times.

Time of incubation (min.)	Enzyme activity remaining after incubation at pH 5·9 and 48·5°		Enzyme activity remaining after incubation at pH 5·0 and 48·5°	
	A	*B*	*A*	*B*
0	100	100	100	100
27	55·5	28·5	—	—
40	—	—	35·5	29
44	36	10·8	—	—
60	26·5	6·6	—	—
70	—	—	18	14
107	—	—	10	6·5

53 day storage in the cold room. These values are only approximate, as they assume similar total recoveries and similar degrees of peak overlapping in the different chromatographic runs.

In the few properties that have been observed, *A* and *B* seem to resemble each other closely. Their pH optima, measured against glycerophosphate in citrate buffers, both lie within the region 5·5–6·0, and, like the enzyme in solution E, neither component is affected by the presence of Mg^{2+} ions. In Table 5 the heat stabilities of *A* and *B* at two pH values are compared. Although they are quite similar, the greater stability of *A* at pH 5·9 may partly explain the changes in the relative amounts of the two which takes place as E is stored.

In order to characterize further the phosphatase and its components a number of observations on their specificity have been made. The criterion used in assigning a given enzyme activity to the phosphatase under investigation is that it shows the same distribution in fractions from the chromatogram as the glycerophosphatase activity. For this purpose the action of fractions obtained from a typical chromatogram have been tested against some phosphates. Fig. 7 shows the distribution of tripolyphosphate and trimetaphosphatase. Both show two peaks corresponding to the glycerophosphatase peaks; but although *A* and *B* seem to have a similar ratio of activity against trimetaphosphate and against glycerophosphate, *A* seems to be relatively more active against tripolyphosphate. Since the measurement of enzyme activities with single samples from each fraction is not strictly quantitative, this conclusion has been confirmed by the more exact method of measuring enzyme activity. Table 6 shows that, relative to their action on glycerophosphate, the enzyme of peak *A* is 2–3 times as active against tripolyphosphate as is that of peak *B*.

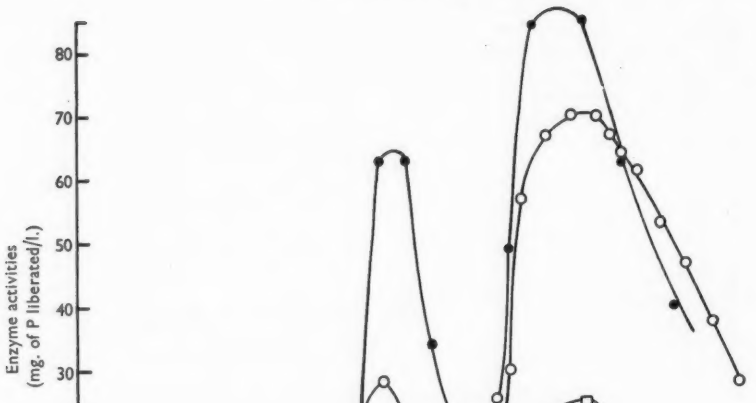

Fig. 7. Polyphosphatase activity in the eluate from a chromatogram. A portion (10 ml.) of fraction E (1750 units of glycerophosphatase) was chromatographed and samples of the eluate fractions (0·2 ml.) were tested for glycerophosphatase (○), tripolyphosphatase (●) and trimetaphosphatase (□). Enzyme activities were estimated as described, except that the buffer was 50 mM citrate (pH 6), and the incubation period was 2 hr.

Table 6. *Ratio of polyphosphatase activities in the chromatographed enzyme components*

Glycerophosphatase, tripolyphosphatase and trimetaphosphatase were estimated in the enzyme components *A* and *B* by the method described, except that the buffer used was 75 mM citrate (pH 6) throughout.

Expt.	Enzyme peak	Tripolyphosphatase / Glycerophosphatase	Trimetaphosphatase / Glycerophosphatase
1	*A*	3·16	0·35
	B	1·44	0·31
2	*A*	3·5	—
	B	1·05	—
3	*A*	4·0	—
	B	1·26	—

The distribution of ATP-ase and ADP-ase among the fractions from the chromatographic column also shows two peaks which correspond to those of glycerophosphatase. Although the ratio of the ATP-ase activity of the two components is similar to the ratio of their glycerophosphatase activity, the second is about half as active as the first towards ADP. None of the fractions had any perceptible activity in liberating orthophosphate from tetrametaphosphate, or phenol from diphenyl phosphate.

It has been suggested (Ledoux, 1953, 1954*a*, *b*) that the multiple nature of pancreatic ribonuclease, as revealed by chromatographic methods, is due to different oxidation–reduction states of four –SH groups in the ribonuclease molecule. Although the hypothesis that ribonuclease has any –SH groups which can affect either its enzymic activity on its chromatographic properties has by no means been universally accepted (e.g. Anfinsen, Redfield, Choate, Page & Carrol, 1954; Rabinovitch & Barron, 1955; Gawron, Keil & Glaid, 1956; Holden & Pirie 1955*b*; Craddock & Dalgliesh, 1955) it seemed worth while to ascertain whether the pea phosphatase is an –SH enzyme. Consequently the action of a number of compounds recommended by Barron (1951) has been investigated on the glycerophosphatase activity. Iodoacetic acid, iodosobenzoic acid and *p*-chloromercuribenzoate had no inhibitory action in concentrations up to $0{\cdot}25 \times 10^{-2}$ M when tested in citrate, maleate or veronal-acetate buffers either with fraction E or with the chromatographed enzyme. Nor was any inhibition brought about by incubating the enzyme with the

–SH reagents for periods up to 30 min. before adding the glycerophosphate. It is concluded therefore that the enzyme does not contain any –SH groups that are necessary for its phosphatase activity.

DISCUSSION

Although the hydrolysis of the poly- and metaphosphates containing three or more phosphate groups has been reported a number of times with extracts of higher plants, in only a few of these have there been any observations on the specificity of the enzymes involved. Thus Yoshida (1953) has produced some evidence that the metaphosphatase of rice bran, estimated by its action on Graham's salt (sodium hexametaphosphate), can be partially separated from the glycerophosphatase activity of his preparations, and Rothenbach & Hinkelman (1954) have prepared extracts of germinated and ungerminated barley which will liberate orthophosphate from hexametaphosphate and trimetaphosphate. The metaphosphatase activity of pea extracts described in the present paper is almost certainly associated with an unspecific phosphatase. Although it does not attack diphenyl phosphate, the enzyme cannot be strictly regarded as a phosphomonoesterase, as it liberates orthophosphate from trimetaphosphate, a reaction involving the splitting of diphosphoester as well as monophosphoester bonds.

Trimetaphosphatase activity has been reported in moulds and animal tissues (Malmgren, 1952), in yeast extracts (Meyerhof, Shatas & Kaplan, 1953; Mattenheimer, 1953, 1956*a*) and in extracts of germinated and ungerminated barley (Rothenbach & Hinkelman, 1954). Only the yeast enzyme has been characterized (Kornberg, 1956; Mattenheimer, 1956*b*, *c*) and shown to be able to convert the cyclic trimetaphosphate into the linear tripolyphosphate. This enzyme is therefore a cyclophosphatase, in the terminology of Mattenheimer (1956*c*), having phosphodiesterase but not phosphomonoesterase activity. In view of this, and of the usual specificity of phosphatases for either phosphodiesters or phosphomonoesters (see Roche, 1950), the splitting of trimetaphosphate by the pea enzyme has been given a little consideration. One possible alternative explanation is that two enzymes are involved, although the distribution of the activity in the eluate from the chromatogram argues against this. It also seems unlikely that the trimetaphosphate is converted non-enzymically into tripolyphosphate on incubation and that this is the compound broken down by the enzyme, since incubation of trimetaphosphate at 37° and at any pH between 3·5 and 7·8 does not cause a detectable increase in the amount of acid groups which dissociate between pH 5 and 7. We intend to study this apparent association of phosphomonoesterase and phosphodiesterase activity, and to ascertain whether this ability to degrade trimetaphosphate distinguishes the pea enzyme from other unspecific phosphomonoesterases.

None of the pea extracts examined had any power to liberate orthophosphate from tetrapolyphosphate, or to convert the latter into a compound which could be degraded to orthophosphate by the enzyme described in this paper. Tetrametaphosphatase activity has been detected in yeast extracts (Mattenheimer, 1956*a*, *b*, *c*), where it converts tetrametaphosphate into orthophosphate by way of tetrapoly-, tripoly- and pyro-phosphate.

The appearance of two components in the chromatography of pea phosphatase is not an artifact caused by the chromatographic process or the presence of trace amounts of metal ions in the solution. There are left, therefore, two main explanations of their appearance. In the first place they may represent completely different enzymes with distinct chemical and physical structures, and perhaps different physiological functions and cellular distributions. An argument against this is the similarity of the component enzymes. They have the same pH optimum against glycerophosphate, are not affected by Mg^{2+} ions or chelating agents, have similar heat stabilities, and the differences between the specificities of the two are only quantitative ones. The alternative explanation, more in keeping with these similarities, and which has been used to explain the chromatographic heterogeneity of other biologically active proteins, is that the two components represent different forms of the same protein molecule.

The other proteins that have been resolved chromatographically into two or more active components are bovine pancreatic ribonuclease (Martin & Porter, 1951), lysozyme (Tallan & Stein, 1953) and insulin (Boardman, 1955). Recently Tanford & Hauenstein (1956) have shown by titrimetric methods that the main, if not the only, difference between the two forms of ribonuclease is the presence of an extra free carboxyl group in one of them. It is suggested that this difference is due, not to the incorporation of an extra amino acid into the ribonuclease molecule, but to the hydrolysis of an amide grouping. The two main components of insulin also differ by the presence of an extra amide group (Harfenist, 1953) and, although it has not been proved, the same suggestion has been made for lysozyme (Tallan & Stein, 1953).

It may well be that a similar explanation holds for the two compounds of pea phosphatase and that they are both derived from the same protein molecule, and differ only in one chemical group. On the other hand, one may represent a much more drastically altered form of the parent protein. There

is evidence from the enzymic digestion of pepsin (Perlmann, 1954), mercuripapain (Hill & Smith, 1956), ribonuclease (Kalnitsky & Rogers, 1955) and the hypophyseal growth hormone (Li, Papkoff, Fønss-Bech & Condliffe, 1956) that proteins may undergo extensive degradations without there being corresponding alterations in their biological activities. Whether the two components of the pea phosphatase represent different states of the same protein, and, if they do, whether the differences exist *in vivo* or are produced during the extraction and purification procedures, are questions for further work.

SUMMARY

1. Pea extracts have a hexametaphosphatase activity which is closely associated with an unspecific phosphatase. A variety of procedures, including protein precipitations, heat denaturation, adsorption and ion-exchange chromatography do not separate these activities or appreciably alter their ratio.

2. The phosphatase liberates orthophosphate from tripolyphosphate, trimetaphosphate, β-glycerophosphate, and adenosine di- and tri-phosphate, but not from tetrametaphosphate. It does not liberate phenol from diphenyl phosphate.

3. The phosphatase is inhibited by fluoride and molybdate but not by a variety of compounds that combine with –SH groups.

4. Ion-exchange chromatography of the phosphatase, with a buffer of higher pH as eluting agent, reveals two distinct enzymically active components. Each component can be separated from the other and rechromatographed as a single peak.

5. The two components are very similar in properties. They have the same pH optimum, are not affected by Mg^{2+} ions or chelating agents, have similar heat stabilities and differ only quantitatively in their specificities.

I wish to thank Dr J. E. Such of the Research Department of Albright and Wilson Ltd. for the gift of the trimeta-, tripoly- and tetrameta-phosphates, and Dr C. Bloomfield for the loan of his fraction collector.

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The Metabolism of Compounds Related to Ethanethiol

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Several workers (Pianto, 1953; Brown *et al.* 1954; Kushner *et al.* 1955; Davies *et al.* 1956) have shown that ethanethiol and compounds closely related to it are strikingly effective antitubercular agents *in vivo* in several species of animal. The effect is confined to the ethanethiol series and is not shown by homologous thiols nor by any of the usual biological ethylating agents. It is noteworthy that *in vitro* the antitubercular activity of ethanethiol is small and non-specific, i.e. no greater than that of homologous thiols.

It seemed possible that the failure of ethanethiol to show activity *in vitro* might be explained by its conversion *in vivo* into a metabolite which was the active antitubercular agent. A metabolic study has therefore been carried out with, in the first instance, compounds related to ethanethiol labelled with ^{35}S. Diethyl disulphide, *S*-ethyl thiolbenzoate and *SS'*-diethyl dithiol*iso*phthalate were chosen for experiments on mice or guinea pigs, because these compounds were known to be potent antitubercular agents in the selected species.

EXPERIMENTAL

Preparation of compounds. Diethyl [^{35}S]disulphide was prepared by oxidation with solid I_2 of a solution of [^{35}S]-ethanethiol in 6N-NaOH, and ethyl [^{35}S]thiolbenzoate by benzoylation of a similar solution. Both compounds were purified by distillation. At the time of preparation, their activity was 31 mc/mole.

[^{35}S]Ethanethiol in NaOH was methylated with dimethyl sulphate; the resultant ethyl methyl [^{35}S]sulphide was dissolved in $CHCl_3$ and oxidized with NaOCl solution at pH 8. The ethyl methyl [^{35}S]sulphone was extracted and distilled; its activity at the time of preparation was 2·2 mc/mole.

SS'-Diethyl dithiol*iso*phthalate was prepared by the reaction of ethanethiol with *iso*phthaloyl chloride (Davies *et al.* 1956).

Measurement of radioactivity. Tissues were homogenized in water (usually 1 ml./g. of tissue) and the paste was spread evenly in polythene planchets 2·5 cm. in diameter. The planchets were placed immediately below the thin mica window of a Geiger–Müller tube and the count was recorded. Liquids were counted similarly. The amount taken was sufficient to give conditions of 'infinite thickness'. The results were recorded as counts/min. calculated for the undiluted tissue or liquid. The time of counting was sufficient to give a result of count corrected for background to an accuracy of 6% at 95% confidence limits. The methods of sample preparation were not thought to justify greater accuracy in counting.

In the experiments to be described, the amount of radioactivity administered is recorded in μc calculated from the initial activity of the source and the known rate of decay. Measurements are given in counts/min. calculated for the undiluted fluid or tissue. Under the conditions used, 1 μc/ml. of solution gave about 3000 counts/min.

Paper chromatogram strips 2 cm. in width were either cut into 2 cm. squares which were counted separately in planchets with an end-window tube and scaling unit, or the uncut strips were moved in 1 cm. steps past a tube having a mask pierced with a 2 cm. × 1·5 cm. rectangular opening (giving an overlap of 0·5 cm. for each section counted) and the counts recorded on a ratemeter. The maximum integrating time (140 sec.) of the ratemeter set a limit to the minimum amount of radioactivity detectable on the paper of about 8 counts/min. above background. With the scaling unit lower activities were detectable, but in practice the convenient length of time available for counting limited the minimum detectable level to about 3 counts/min.

Treatment of animals. Compounds for oral administration were prepared as 10% (w/v) dispersions in aqueous solution containing 1·5% of Dispersol OG and 1% of Cellofas WLD (both from Imperial Chemical Industries Ltd.), and were given by catheter. Compounds for subcutaneous injection were given as 25% (v/v) solutions in arachis oil. Animals were placed in metabolism cages and urine and faeces were collected separately. For some experiments mice were confined in a closed glass tank through which air was slowly drawn. The issuing air passed through traps containing permanganate solution.

Extracts of tissues. The tissue was homogenized with water (2 ml./g.) and the suspension was centrifuged. The residue was extracted twice more in a similar way leaving spent residue, *A*; the supernatants were pooled. Trichloroacetic acid (20%, w/v) was added (0·2 ml./ml. of extract) and the precipitate, *B*, removed by centrifuging. The solution was extracted three times with ether; the combined ether extracts, *C*, contained most of the excess of trichloroacetic acid. The aqueous solution, *D*, was concentrated under reduced pressure for paper chromatography. The fractions *A*, *B*, *C*, *D* contained 4, 9, 1 and 86% of the radioactivity respectively.

Concentration of organic metabolites from urine

By removal of urea and salts. Jack-bean meal (1·25 g.) was shaken with a solution of ethanol (15 ml.) and water (35 ml.) for 30 min., and then centrifuged. The supernatant was used as a urease preparation. Urine (100 ml.) from guinea pigs dosed with diethyl [^{35}S]disulphide was warmed to 30°; 5 mg. of KCN was added. Urease solution (50 ml.) at 30° was run in and the mixture was kept stirred; 5N-HCl was

run in as required to keep the pH between 6·3 and 7·3. After 2–3 hr. at 30°, change of pH ceased (acid added was approx. 8 ml.). The solution was acidified and N_2 was passed through to remove CO_2. Van Slyke determinations showed that 98% of the urea had been removed.

The solution was passed through a column containing a mixture of anionic and cationic resins [Zeo-Karb 225 (H), 40 g., and Deacidite FF (OH), 50 g.] to remove the electrolytes. The resultant solution was concentrated under reduced pressure for paper chromatography or countercurrent separation. Of the original radioactivity, 16% was recovered in the solution from the ion-exchange column; a similar activity was found in a sample of the same urine from which sulphate had been removed by addition of Ba^{2+}. Tests on the concentrate showed the metabolic product to be neutral (not absorbed by strong anionic or cationic resins), somewhat volatile and fairly polar. It was only very slightly extractable by ether and its partition coefficient in butanol–water was 0·48. It was stable to acids and alkalis.

A similar method of concentration was used for 20 l. of urine from guinea pigs dosed with inactive diethyl disulphide. In view of the volatility of the metabolite revealed by the experiments with radioactive material, the aqueous concentrate was evaporated to dryness under reduced pressure at the lowest practicable temperature, and the residue distilled. Crystallization of the distillate yielded slightly impure ethyl methyl sulphone; this was, however, more readily obtained by the solvent-extraction method.

By solvent extraction. Guinea pigs (approx. 500 g. body wt.) received a daily subcutaneous dose of *SS'*-diethyl dithio*iso*phthalate (200 mg./kg.) as a 50% (v/v) solution in arachis oil. This compound is well tolerated by the animals. Urine was collected and preserved by addition of $CHCl_3$. The combined urine (18 l.) was extracted with $CHCl_3$ (4 × 30 l.), the extract was filtered and the solvent distilled off. The residue was stirred with water (200 ml.) and ether (20 ml.). After separation, the aqueous layer was further extracted with ether (2 × 20 ml.). The combined ether layers on evaporation gave 2·5 g. of black waxy residue. The aqueous layer was extracted with $CHCl_3$ (5 × 200 ml.) and the solvent distilled off. The residue was distilled at 0·05 mm. giving fractions with b.p.'s 60–70° (0·50 g.), 70–105° (0·08 g.) and 105–120° (0·15 g.). Only a little non-volatile residue remained. The lowest-boiling fraction (m.p. 28°) was crystallized from methanol at −20°, giving 0·11 g. (m.p. 33·5°). Further crystallization from ether gave long colourless needles (0·08 g.), m.p. 33·5°, unchanged by admixture with ethyl methyl sulphone, m.p. 33·5°. [Beckmann (1878) reported 36°.] (Found: C, 33·0; H, 7·4; S, 29·5, 29·9. Calc. for $C_3H_8O_2S$: C, 33·3; H, 7·4; S, 29·7%.) No pure substance was isolated from the higher-boiling fractions.

Comparison of metabolites from urine with ethyl methyl sulphone

Ethyl methyl [^{35}S]sulphone was compared with crude urines from three species of animal dosed with diethyl [^{35}S]disulphide and with a purified concentrate of guinea-pig urine by paper chromatography in various solvents. The same concentrate was also examined by countercurrent distribution.

Paper chromatography. The downward method was used with Whatman no. 541 paper and the following solvents: (*a*) propan-1-ol, 280 ml., water, 250 ml., *n*-butanol to 1 l.; (*b*) $CHCl_3$, 200 ml., water, 200 ml., acetic acid to 1 l.; (*c*) 80% (v/v) dioxan in water. Aqueous phenol was unsuitable.

Countercurrent distribution. Ethyl methyl [^{35}S]sulphone (1 g.) was dissolved in water saturated with $CHCl_3$ and distributed through 49 transfers between $CHCl_3$ and water in a Craig machine (volume of upper and lower phases, 25 ml.). The contents of each tube were removed and the mixture was evaporated under reduced pressure until the $CHCl_3$ had been removed. The volume was then adjusted to 25 ml. with water and the aqueous solution was counted.

Urine concentrate (25 ml.) free from urea and electrolytes (activity, 425 counts/min. in 2·5 cm. planchet) was distributed exactly as for the ethyl methyl sulphone. The contents of each tube were concentrated, adjusted to 5 ml. and counted. Results appear in Fig. 4.

Examination of metabolite B. Solution from tubes 34–38 in the Craig separation described above containing metabolite *B* (see Fig. 4) was concentrated to 2 ml. Its behaviour on paper chromatography was compared with that of ethyl methyl sulphone and of ethyl methyl sulphoxide. The position of the (non-radioactive) sulphoxide spot was revealed by spraying with permanganate solution (Fig. 3).

The partition coefficient of ethyl methyl sulphoxide between $CHCl_3$ and water was estimated by equilibration and subsequent evaporation of the $CHCl_3$ layer and determination of the weight of residue. It was also estimated in both layers by oxidation with excess of permanganate and back titration of the excess. Neither method was very precise but the results were in rough agreement. Partition coefficient (K) by weight was 4·9, and by permanganate titration 4·6.

RESULTS

Preliminary experiments with mice showed that the labelled compounds were rapidly absorbed after oral or subcutaneous administration, and distributed evenly throughout the body tissues without any notable local concentration; a typical result is shown in Table 1. Excretion of radioactivity occurred mainly via the kidney. Faecal excretion was small, but appreciable excretion (14% of the dose in one experiment) occurred in the breath, probably as ethanethiol. In view of the volatile

Table 1. *Radioactive counts of tissues from mice receiving diethyl* [^{35}S]*disulphide*

Three 25 g. mice each received subcutaneously 10 mg. (2 μc) of diethyl[^{35}S]disulphide followed after 18 hr. by an equal dose; tissues were taken 4·5 hr. after the second dose.

Tissue	Combined wt. of organs (g.)	Radioactivity (counts/min.)
Alimentary tract (with contents)	12·6	183
Liver	4·69	126
Kidney	1·54	124
Spleen	0·57	92
Heart	0·50	94
Lung	0·82	128
Blood	1·3	118

nature of the thiol it was difficult to obtain accurate balances of radioactivity administered and recovered, but over a period of 24 hr. the urine of both mice and guinea pigs yielded 40–65 % of the radioactivity dosed in the form of relatively non-volatile compounds together with an undetermined amount of ethanethiol.

The course of urinary excretion of radioactivity in the guinea pig is shown in Fig. 1. A logarithmic plot of the fraction of the total excreted radioactivity against time gave a linear curve which was identical for diethyl disulphide and for ethyl thiolbenzoate. This result suggests that rate of excretion is governed by a single process; there is no evidence of rapid excretion of one product followed by slower excretion of another. In excretion of ^{35}S the two thiol-generating compounds apparently behaved similarly. Half the total ^{35}S was excreted in about 10 hr.

The nature of the radioactive compounds in mouse tissues was investigated by aqueous extraction and precipitation of protein. Most of the radioactivity remained in the aqueous solution after removal of the excess trichloroacetic acid by ether extraction. The nature of radioactive material taken down with the protein precipitate or not extracted from tissues by aqueous extraction (10–15 % of the total) was not determined. Other extraction procedures suggested that part of it was extractable into ether from aqueous suspension. The aqueous extract representing the major part of the relatively non-volatile radioactive material of the tissues was concentrated and examined by paper chromatography. At least 80 % of the radioactivity from muscle and visceral extracts, excluding the intestines, was found in a single peak in several different solvent systems (Fig. 2). A similar peak was observed with intestinal extracts, though considerable radioactivity remained at the origin.

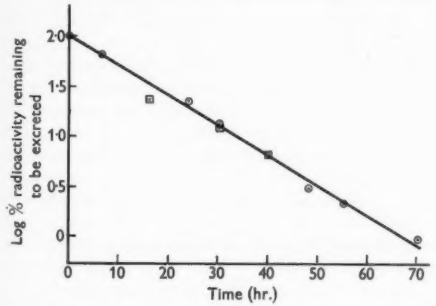

Fig. 1. Urinary excretion of radioactivity from a guinea pig (540 g.) dosed orally with ^{35}S-labelled compounds. ⊙, Ethyl thiolbenzoate, dose 200 mg. (27 μc); ⊡, diethyl disulphide, dose 100 mg. (40 μc).

When mouse urine was examined, a high activity was found at the origin and most of the remainder in a single peak identical with that found in the tissues (Fig. 2). The material which did not migrate in paper chromatography was precipitated from acid solution by Ba^{2+} ions and was identified as sulphate. Urine from guinea pigs dosed with labelled derivatives of ethanethiol resembled that from mice when examined in the same way, but rabbit urine showed two distinct and nearly equal peaks of radioactivity apart from the usual high level of sulphate.

The isolation and identification of ethyl methyl sulphone from urines of guinea pigs dosed with either diethyl disulphide (labelled and unlabelled) or diethyl dithiol*iso*phthalate has been described above. It remained to establish the fact that ethyl methyl sulphone was the metabolite revealed by radioactive labelling. Radioactive sulphone was

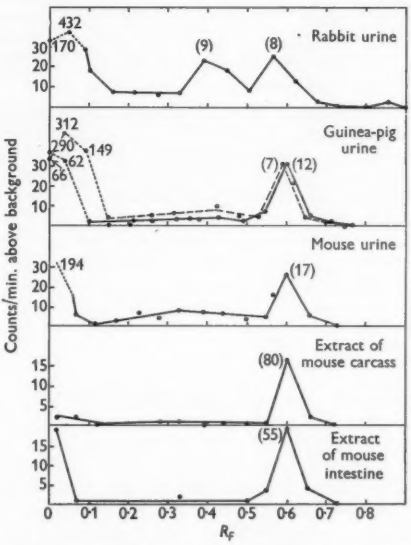

Fig. 2. Paper chromatography of urine and tissues from animals dosed orally with ^{35}S-labelled compounds. Rabbit (1·5 kg.); received 300 mg. (120 μc) of diethyl disulphide; 20 hr. urine. Guinea pig: ●—●, diethyl disulphide, 20 hr. urine; ●– – –●, ethyl thiolbenzoate, 6·5 hr. urine; wt. and dose as in Fig. 1. Mice (25 g.): each received 4 mg. (2 μc) of diethyl disulphide; urine and tissues were taken 4·5 hr. after dose. Amounts applied to paper: urine, 10 μl.; tissues, extract equivalent to 100 μg. moist wt. Developing solvent, butanol–propanol–water. Figures in parentheses show approx. percentage of total radioactivity in the peak. Dotted parts of curve are not to scale; counts/min. are shown by figures. All counts were made by scaler on sections of paper.

compared with radioactive concentrates of urine by paper chromatography and countercurrent distribution. The materials in three different solvent systems gave spots almost identical in position, except that those from the urine concentrate appeared to be rather more spread (Fig. 3). Distribution of labelled ethyl methyl sulphone between chloroform and water in a 50-tube Craig machine gave a single peak agreeing well with the theoretical distribution of a substance having K 0·69. The urine concentrate showed a very similar peak together with a second smaller one. The distribution of radioactivity could be well represented by the summation of theoretical curves for two substances having K 0·75 and 2·70 (Fig. 4). The slight difference in partition coefficient between the first peak and that of ethyl methyl sulphone is not regarded as significant. It only represents a difference of one tube in the series and may well be caused by the slight emulsification which occurred in separating the urine concentrate. The results indicate that ethyl methyl sulphone is certainly the main organic metabolite of ethanethiol derivatives in urine. There is also a second metabolite, B, present to the extent of 40% of the sulphone.

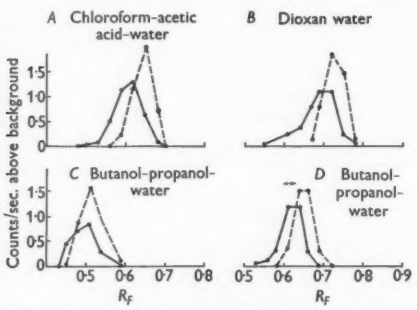

Fig. 3. Paper chromatography of ^{35}S-labelled metabolites of urine. Curves A, B, C: ●—●, guinea-pig urine after removal of urea and electrolytes; ●- - -●, ethyl methyl sulphone, 150 μg. (0·003 μc); developing solvents as indicated. Curve D: ●—●, metabolite B from countercurrent separation; ●- - -●, sulphone as in curves A, B, C; ↔, position of ethyl methyl sulphoxide spot. All counts were made on strips with ratemeter.

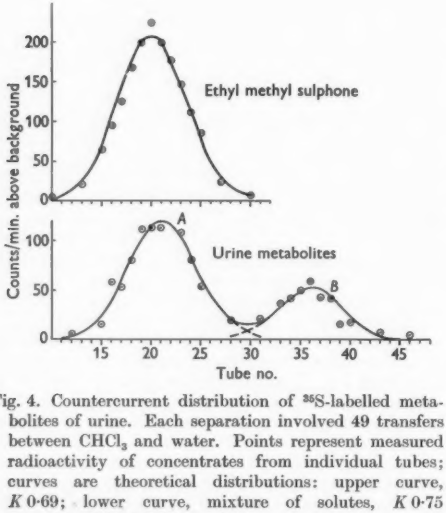

Fig. 4. Countercurrent distribution of ^{35}S-labelled metabolites of urine. Each separation involved 49 transfers between $CHCl_3$ and water. Points represent measured radioactivity of concentrates from individual tubes; curves are theoretical distributions: upper curve, K 0·69; lower curve, mixture of solutes, K 0·75 (1 part) and K 2·70 (0·4 part).

Metabolite B is evidently not readily separable from the sulphone by paper chromatography in three quite diverse solvents. It is not certain whether it occurs in the tissues, since the only comparison between the metabolites in urine and tissues was made by paper chromatography. Its nature has not yet been established, and it is not known whether it is a constituent of the higher-boiling fraction obtained in the extraction of non-radioactive urine. Paper chromatography showed that ethyl methyl sulphoxide could just be separated from the sulphone, but could hardly be separated from metabolite B (Fig. 3). However, approximate determinations of the partition coefficient of the sulphoxide between chloroform and water gave values considerably different from those found for metabolite B in the countercurrent separation. The question of the identity of this product thus remains unsolved.

Neither ethyl methyl sulphone nor a crude mixture containing metabolite B was significantly effective in protecting mice from tubercular infection.

DISCUSSION

Few investigations have been made of the metabolism of alkyl thiols. Canellakis & Tarver (1953) showed that when methanethiol is given intraperitoneally to rats, the sulphur appears as sulphate in the urine, and is not significantly incorporated into the methionine or cysteine of liver protein. The carbon atom is available for incorporation into the methyl groups of choline and methionine and into the β-carbon atom of serine. According to the present experiments with three animal species the sulphur of ethanethiol is excreted mainly as inorganic sulphate. However, organic metabolites account for 10–20% of the sulphur excreted, and the chief of these in the mouse and guinea pig is ethyl methyl sulphone. In tissues the sulphone preponderates, probably because of a more rapid excretion of sulphate; it is also possible that the breakdown to sulphate occurs in the kidney itself.

The most probable course of the methylation and oxidation involved in the conversion of ethanethiol into ethyl methyl sulphone would appear to be a preliminary conversion into ethyl methyl sulphide, followed by oxidation to the sulphone. Evidence for biological methylation of sulphur compounds related to ethanethiol has been presented by Challenger & Rawlings (1937), who showed that the mould *Scopulariopsis brevicaulis* converts diethyl disulphide into ethanethiol and ethyl methyl sulphide. At least one example of the oxidation of a sulphide to a sulphone *in vivo* has been reported. Rose & Spinks (1948) showed that *p*-methylthioaniline was readily converted into the corresponding sulphone by mice, rats or rabbits.

Schlenk & Palma (1955) proposed the reaction

$$(CH_3)_2\overset{+}{S}.CH_2.CH_2.CH(NH_2).CO_2H + HS.CH_2.CH_2.CH(NH_2).CO_2H \rightarrow 2CH_3.S.CH_2.CH_2.CH(NH_2).CO_2H$$

to explain the observation that a mixture of homocysteine and methionine *S*-methiodide can replace methionine as a source of methylthioadenosine in yeast, although neither is nearly as effective separately. A similar type of reaction with a sulphonium compound could be imagined as a mechanism for the methylation of ethanethiol:

$$C_2H_5.SH + RR'\overset{+}{S}.CH_3 \rightarrow C_2H_5.S.CH_3 + R.S.R' + H^+.$$

Sulphonium compounds occur in living tissues, and one, *S*-adenosylmethionine, has already been indicated as an intermediate in biological methylation (Cantoni, 1953); the methyl sulphonium compound corresponding to methionine has been found in plant tissues (Challenger & Hayward, 1954; McRorie *et al.* 1954). Another way in which alkyl sulphides can arise by an enzymic process is illustrated by their formation in a lichen preparation from sulphonium compounds of a thetin type (Challenger & Liu, 1950). It would, however, seem preferable not to postulate the formation of an ethyl sulphonium compound of the type $C_2H_5.\overset{+}{S}(CH_3)R$ since it is doubtful whether the radioactive label would be retained in the formation of such a derivative, and, further, the intermediate might be expected to function like ethionine as a biological ethylating agent. No evidence has ever been observed of the ethionine type of toxicity (Stekol & Weiss, 1949) when compounds related to ethanethiol have been administered in frequent high doses to mice or guinea pigs over periods of several weeks. It is, however, not certain how sensitive these species may be to interference with the normal methyl metabolism.

It seems improbable that the carbon of ethanethiol can be available for incorporation into compounds in the same way as that of methanethiol (Canellakis & Tarver, 1953), but knowledge of the fate of carbon atoms released in the formation of sulphate must await the result of experiments with carbon-labelled material. It is not known whether sulphones occur in the metabolism of the normal sulphur compounds of the body, but Ruzicka, Goldberg & Meister (1940) isolated dimethyl sulphone from ox blood. Experience suggests that such chemically inert and rather volatile compounds may easily be overlooked in metabolic studies.

The features of ethanethiol metabolism revealed in these experiments provide no explanation of its specific antitubercular action *in vivo*, since neither ethyl methyl sulphone nor the unidentified metabolite shows this activity. The possibility remains that some minor metabolite, not revealed by the separation methods used, could be the effective agent. However, almost every possible oxidative metabolite and derivative of ethanethiol has been prepared and tested (Davies *et al.* 1956), and none was active unless easily convertible into the thiol. Present evidence favours the view that the antitubercular activity is due to ethanethiol itself. This compound is certainly very quickly removed from circulation by metabolism, and the greater relative effectiveness of certain derivatives of the thiol (notably the thiol esters), especially by subcutaneous injection, probably reflects a comparatively slow release of the parent substance. It is known that early multiplication of the tubercle bacillus occurs largely in the macrophages, and the specific inhibitory action of ethanethiol may result from some peculiarity of local metabolism in the macrophage or in the micro-organism growing in this special environment.

SUMMARY

1. Diethyl disulphide and *S*-ethyl thiolbenzoate (labelled with ^{35}S) have been given to mice and guinea pigs and the radioactivity of tissues and excreta measured. Radioactivity is rapidly and fairly uniformly distributed throughout the body.

2. Radioactivity is excreted mainly in the urine; in a guinea pig 50% of the total urinary excretion occurred in about 10 hr. Little radioactivity is excreted in the faeces, but a substantial amount in the breath in the form of ethanethiol.

3. Sulphate accounts for 80–90% of the radioactivity in urine. Two organic metabolites are also present, one of which is ethyl methyl sulphone; the other has not been identified.

4. The major part of the radioactivity found in the body tissues of dosed mice comes from ethyl methyl sulphone, perhaps in association with the unidentified organic metabolite.

5. Neither ethyl methyl sulphone nor a crude mixture containing both organic metabolites showed any marked antitubercular activity when tested *in vivo*.

The author is indebted to Mr G. E. Davies and Dr A. R. Martin for dosing and care of animals, and to Mr J. C. B. Smith for technical assistance. Dr G. W. Driver kindly provided the *SS'*-diethyldithiol*iso*phthalate.

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Ion Transport and Respiration of Isolated Frog Skin

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In the search for the mechanism of active ion transport by cells the hypothesis was early introduced that such transport might be causally linked to the transference of electrons in the oxidation–reduction reactions of the cells. The concept of oxidation–reduction reactions as sources of bioelectric phenomena in cells and tissues was first introduced by Lund in 1928. Ten years later this concept was applied to the movement of ions and secretory processes by Friedenwald & Stiehler (1938) and Stiehler & Flexner (1938). Subsequently, this hypothesis was revised and extended by a number of workers studying ion transport (Lundegårdh, 1939; Robertson & Wilkins, 1948*a*, *b*; Conway & Brady, 1948; Crane & Davies, 1948; Patterson & Stetten, 1949; Rehm, 1950). These views have been incorporated by Davies & Ogston (1950) into their mechanism I and by Conway (1951, 1952, 1953, 1955) who has conveniently termed this hypothesis the 'redox pump' theory of ion transport.

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Basically these hypotheses state that the following two reactions occurring in aerobic metabolism are spatially separated within the cell:

$$4Fe^{3+} + 4H = 4Fe^{2+} + 4H^{+},$$
$$4Fe^{2+} + O_2 + 2H_2O = 4Fe^{3+} + 4OH^{-}.$$

Fe^{3+} and Fe^{2+} are the oxidized and reduced forms of cytochrome iron; hydrogen atoms arise from the substrate of metabolism. The electrons picked up from the hydrogen atoms in the first reaction are considered to be passed along an oxidation–reduction chain to another site in the cell where they are finally received by molecular oxygen. The hydrogen ion formed in these reactions would be available for secretion as such, or for exchange with cations outside the cell. The same reasoning can be applied to make this system transport anions. Oxidation–reduction systems which do not contain iron have been similarly considered (Patterson & Stetten, 1949).

As pointed out by Lund & Stapp (1947) and Robertson & Wilkins (1948*b*), this hypothesis has a quantitative limitation which may be tested experimentally. A maximum of four electrons, or

univalent ions, may be transported per molecule of oxygen consumed.

In the present study this limitation was investigated by measuring simultaneously active ion transport and oxygen consumption by the isolated frog skin. Ion transport was measured electrically by the short-circuit-current method of Ussing & Zerahn (1951). Oxygen consumption was determined by a polarographic technique with vibrating platinum micro-electrodes.

The results show that, at least in this tissue, the number of ions actively transported per molecule of oxygen consumed considerably exceeds this critical value. This indicates that the 'redox' hypothesis as proposed by Conway (1954, 1955) and Lundegårdh (1954), in which oxygen is the electron acceptor, is quantitatively insufficient to account for the active transport of ions by this tissue.

METHODS

Experiments were done on the ventral-abdominal skin of male frogs (*Rana temporaria*) weighing 20–25 g., during the winter and early spring months. The frogs were stored at 4° and brought to room temperature for 1–5 days before use. They were force-fed with meal worms on removal from the refrigerator. At the start of an experiment the ventral-abdominal skin was washed with soap and water and rinsed, and the animal killed and pithed. The ventral skin was quickly dissected and, after rinsing in frog Ringer solution, mounted into a chamber similar to that described by Ussing & Zerahn (1951).

In order to ascertain that the chamber was free from bubbles during an experiment a transparent material was essential. Both Perspex and glass chambers were used. Fig. 1 shows a diagram of the chamber together with the accessory equipment necessary for the measurement of short-circuit current and O_2 consumption. The frog skin was mounted at *A* between the two separate halves of the chamber, which were then clamped together by the large screws at either end, making the system water and gas tight. The area of frog skin within the chambers was 3·14 cm.^{2}. Four bridges, consisting of polyethylene tubing of 1·3 mm. internal diam. filled with 2% (w/v) agar made up in 0·11 M-KCl solution, were used to measure the potential difference across the membrane and to apply the external counter-potential which reduces the membrane potential to zero during the measurements of short-circuit current. The Pt micro-electrodes were made of short lengths of Pt wire, 0·25 mm. in diam., sealed into one end of a glass tube approximately 4 cm. long. The Pt wire was cut off and polished flush with the tip of the glass tube. The tube was filled with Hg and sealed with a small brass cap through which a stainless-steel wire made contact with the Hg. This Pt micro-electrode was forced through a tightly fitting rubber stopper in the top of the chamber so that about 1·0 cm. of the glass tube projected freely into the chamber. The portion of the electrode protruding above the chamber was vibrated laterally at 50 cyc./sec. so that the platinum tip inside the chamber was displaced 3–5 mm. To ensure a constant rate and amplitude of vibration during the measurement a Mickle shaker (H. Mickle, 4, Ormond Drive, Hampton, Middlesex, England) was converted for this purpose. The Pt micro-electrode was connected to the negative pole of a polarograph (Cambridge Polarograph, Pen-Recording Type, Cambridge Instrument Co. Ltd., Grosvenor Square, London) and the circuit was completed through an agar–0·11 M-KCl bridge of polyethylene tubing with one end in the chamber and the other dipping into the 0·11 M-KCl of an Ag–AgCl electrode. The volume of each half-chamber was determined by weighing with Hg. The volumes of the left-half chambers were 3·87 and 3·19 ml., and those of the right-half chambers 3·68 and 3·66 ml. for the Perspex and glass chambers respectively.

A rapidly moving noble-metal micro-electrode has been applied and validated for the determination of dissolved O_2 by other workers (Marsh, 1951; Longmuir, 1954; Warshowsky & Schantz, 1954; Chance & Williams, 1955). With an applied potential of 0·65 v across the Pt and Ag–AgCl electrodes, only molecular O_2 in the Ringer solution will remove electrons from the Pt cathode. The current measured through this system is proportional to the O_2 concentration.

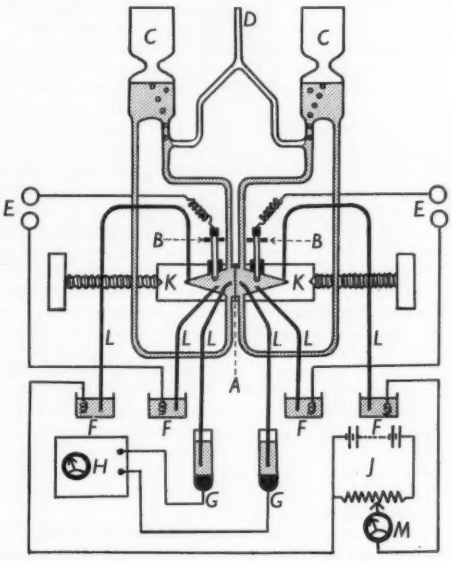

Fig. 1. Diagrammatic sketch of the apparatus. The frog skin *A* separates the two halves of the chamber *K*, which are clamped between two set screws. A Perspex cuff *B* from the vibrator holds the platinum micro-electrodes which are connected with the polarographs *E*. The polarographic circuit is completed through a silver–silver chloride electrode *F* and a 0·11 M-KCl–agar bridge *L* with the chamber. The fluid in the chamber is circulated and aerated in the reservoirs *C* by gas entering from *D*. Two calomel electrodes *G* connected to tube potentiometer *H* are used to measure the potential across the skin. An external potential source *J* supplies the potential for short-circuiting the skin and the short-circuit current is read from the micro-ammeter *M*.

The Pt micro-electrode was vigorously vibrated to eliminate any effect of variations in the 'diffusion cone' of O_2 in the layers of medium immediately adjacent to the Pt electrode surface and also to provide adequate stirring of the medium on each side of the skin during the periods of measurement when the contents of the chamber were sealed off.

The method of Harris & Barclay (1955) was used to determine the exact relationship of O_2 tension to the diffusion current. The electrode system was placed in a glass chamber which could be evacuated, allowing measurements of O_2 tension and diffusion current to be made in a small volume of Ringer solution. Stepwise additions of 100% O_2 or room air were then introduced, the gas pressure was measured, and the diffusion current in the enclosed solution in equilibrium with the gas phase measured. Fig. 2 shows that with 100% O_2 as the gas phase a linear calibration curve was obtained. As experiments were made with room air as the gas phase, measurements were repeated with room air rather than pure O_2. Fig. 3 shows that a similar linear calibration was obtained over this range of O_2 tensions.

After mounting the frog skin in the chamber each half of the chamber and its aeration reservoir were filled with about 10 ml. of frog Ringer solution (112 mM-NaCl, 1·88 mM-KCl, 2·38 mM-$NaHCO_3$ and 0·89 mM-$CaCl_2$). Care was taken to eliminate all gas bubbles from the chamber. The bathing medium was aerated with room air or other appropriate gas mixture through the bubbling and mixing system described by Ussing & Zerahn (1951). After a period of equilibration with room air, during which a constant O_2 diffusion current was recorded by the polarograph, the inlets and outlets of the chamber were quickly clamped off with small surgical arterial bulldog clamps. The linear decrease in the diffusion current indicates the drop in O_2 concentration in the enclosed volume of fluid in contact with the frog skin during the period of measurement. The 'zero' value for the O_2 diffusion current was obtained by bubbling cylinder N_2 into the chamber to wash out all traces of O_2 from the bathing solution. Essentially all O_2 was first removed from the cylinder N_2 by bubbling the latter through a vanadyl sulphate solution as recommended by Meites & Meites (1948). The quantity of O_2 consumed by the skin was calculated from the known values for the volume and temperature of fluid in each chamber, the solubility of O_2 in Ringer solution and the linear relation between O_2 concentration and diffusion current. Because of the relatively slow rate of diffusion of O_2 through the skin simultaneous measurements of O_2 disappearance were made separately in the medium bathing each side of the skin.

In order to assess the reproducibility of the O_2 measurement by this technique air was mixed with N_2 in a large cylinder to give a final O_2 concentration in the range of that measured in these studies. After each period of measurement the chamber fluid was equilibrated with gas from this cylinder and measurements were made on the O_2 concentration in this cylinder mixture. A total of 131 such measurements was made during the course of the experiments and the coefficient of variation for a single measurement of O_2 concentration was found to be 0·84%. There was no difference between the measurements made simultaneously, but separately, on each side of the skin.

The temperature of the bathing solution was 23–28° in these experiments and was measured before and after each 15–20 min. period. No substrate was added to the Ringer solution. To minimize any O_2 consumption due to the unavoidable presence of bacteria on the frog skin the ventral abdominal skin was washed with soap and water immediately before its dissection and the duration of the experiments was kept down to 2 hr. from the time of mounting the skin. In the last 74 periods of measurement crystalline sodium penicillin (165 units/ml. of Ringer solution) and streptomycin calcium chloride (0·1–0·2 mg./ml. of Ringer solution) were contained in the bathing medium.

Ussing & Zerahn (1951) have shown that, with the same medium on either side of the skin, when an external potential is applied across the isolated frog skin so as to reduce the spontaneous membrane potential to zero, the current

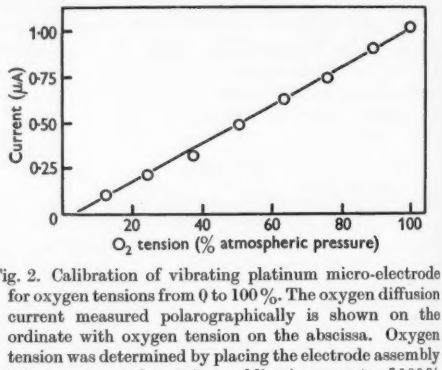

Fig. 2. Calibration of vibrating platinum micro-electrode for oxygen tensions from 0 to 100%. The oxygen diffusion current measured polarographically is shown on the ordinate with oxygen tension on the abscissa. Oxygen tension was determined by placing the electrode assembly into an evacuated container, adding increments of 100% oxygen and measuring the pressure in the closed system after each addition.

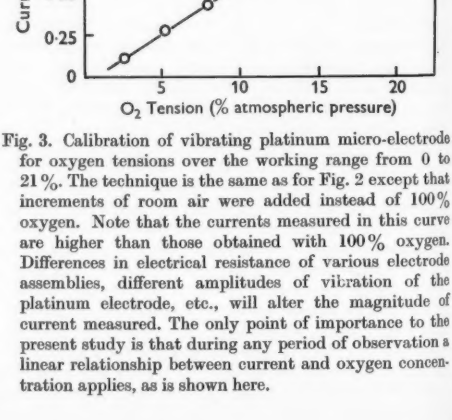

Fig. 3. Calibration of vibrating platinum micro-electrode for oxygen tensions over the working range from 0 to 21%. The technique is the same as for Fig. 2 except that increments of room air were added instead of 100% oxygen. Note that the currents measured in this curve are higher than those obtained with 100% oxygen. Differences in electrical resistance of various electrode assemblies, different amplitudes of vibration of the platinum electrode, etc., will alter the magnitude of current measured. The only point of importance to the present study is that during any period of observation a linear relationship between current and oxygen concentration applies, as is shown here.

measured in the external circuit just equals the active Na transport through the skin. Table 1 shows the equality of net Na transport and short-circuit current through the isolated ventral skin of *Rana temporaria* in 14 separate periods of 60 min. each. Four frogs were used. The techniques of measurement are those described by Ussing (Levi & Ussing, 1949; Ussing & Zerahn, 1951). The results confirm a much larger number of similar measurements made in Ussing's laboratory (Ussing & Zerahn, 1951; Fuhrman, 1952; Ussing, 1954) and by Linderholm (1952). Because of this equality of short-circuit current and active Na transport, the former has been used as the measure of the latter in these experiments. Readings of short-circuit current were made directly from a micro-ammeter at intervals of 2·5 min. during each period and averaged to obtain the mean ion transport during the period.

When posterior-pituitary preparations were tested for their effect on O_2 consumption and short-circuit current they were added to the Ringer solution bathing the inside of the skin after at least two control periods had been obtained. The posterior-pituitary extract used was Pitressin (Parke, Davis and Co. Ltd., Batch No. LS 588 F), of which 2 units was added. Dried whale posterior-pituitary powder (generously made available by Professor H. H. Ussing) and pure Vasopressin (A-V-N-3) and Oxytocin (O-N-3, generously supplied by Professor V. du Vigneaud) were also used. Approximately 1 mg. of the dry powder or 1 unit of the pure hormones was added to the solution bathing the inside of the skin.

Manometric measurements were made of O_2 consumption of frog skin with the standard Warburg apparatus. The ventral-abdominal skins of 15 frogs were divided sagittally into two halves. Each half, with wet weight of about 0·4 g., was placed in a separate flask containing 4·0 ml. of frog Ringer solution without added substrate and 1 unit of Pitressin (0·05 ml.) in the side arm of one flask. After 20 min. for equilibration the Pitressin was tipped into the medium and measurements were made of O_2 uptake at 5 min. intervals for two consecutive 1 hr. periods.

RESULTS

Relationship between ion transport and oxygen uptake. The observed relationship of active ion transport to total oxygen consumption by the isolated frog skin is shown in Fig. 4 as a frequency distribution graph of the number of sodium ions transported per molecule of oxygen consumed in the 120 periods studied. In only five periods was this ratio found to be 4·0 or less. It is apparent that in the majority of experimental periods the number of ions transported per molecule of sodium exceeds the upper limit of 4·0 required by the 'redox' hypothesis, as recently formulated by Lundegårdh (1954) and Conway (1953, 1954, 1955). The mean value for this ratio is 6·82 and the standard error of the mean is ±0·16, indicating that even the mean of this series of observations significantly exceeds a value of 4·0.

Evaluation of experimental error. An attempt has been made to evaluate the magnitude of possible errors which may enter into the determination of this ratio. The coefficient of variation of a single polarographic measurement of oxygen concentration was 0·84% (a standard deviation of 0·16 for a series of 131 measurements on a standard gas mixture containing 19·25% of oxygen). As the oxygen consumption in a usual 15–20 min. period

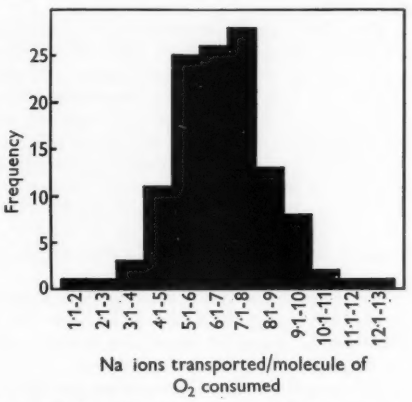

Fig. 4. Relationship of ion transport to total oxygen consumption by the isolated frog skin. The figure is a frequency distribution graph of the results of 120 separate periods of measurements made simultaneously of short-circuit current and oxygen consumption. The ordinate is the frequency at which any given ratio of the number of sodium ions transported/molecule of oxygen consumed was obtained. The values of this ratio form the abscissa. The great majority of values for the ratio are above 4 and thus incompatible with the unmodified 'redox' hypothesis of ion transport.

Table 1. *Comparison of net sodium flux and short-circuit current of isolated frog skin*

Sodium influx was measured with ^{22}Na and outflux with ^{24}Na according to Levi & Ussing (1949). Mean net Na flux is influx minus outflux. Short-circuit current was measured directly as described in text. Values in columns 3–6 are expressed as μequiv. of Na/cm.²/hr.

No. of periods	Duration of each period (min.)	Mean Na influx	Mean Na outflux	*A* Mean net Na flux	*B* Mean short-circuit current
14	60	1·54	0·125	1·42	1·48

Mean difference $(B - A) = +0{\cdot}059$ μequiv./cm.²/hr.; S.E. mean difference $= \pm 0{\cdot}035$ μequiv./cm.²/hr.; hence means do not differ significantly.

was 2–3 % of the original 21 % present, the error in a single measurement of oxygen consumption may be expected to be ± 5–8 %. The volume of the chambers is known by calibration to an accuracy of approximately 0·4 % and this source of error is therefore negligible. To exclude the possibility that oxygen was diffusing from the Perspex into the Ringer solution, giving thereby falsely low measurements of oxygen consumption, additional measurements were made in a glass chamber. The mean and standard error of the ratio of ions transported to molecules of oxygen consumed in 13 periods with the glass chamber was 6·56 ± 0·45 as compared with 6·85 ± 0·17 for the 107 measurements made with the Perspex chambers. As these two series are not statistically different the possibility of a significant diffusion of oxygen from the heavy-walled Perspex chamber is excluded, and the results have been pooled together.

As the inlet and outlet tubes to the chamber could not be clamped off flush with the chamber some error was introduced by oxygen diffusing from the volume of fluid within the narrow tubing into the chamber. The radius of each opening was 1 mm. and the volume of fluid occupying this dead space was found by calibration with mercury to be 2·9 and 4·7 % of the volume of the Perspex and glass chambers respectively. Thus if all the oxygen contained in this trapped volume of fluid were available to the frog skin an error of this magnitude would be introduced.

Another potential source of error was the presence of air bubbles trapped in the chambers during measurements. A careful inspection of the transparent chamber was made with a bright beam of light before each period to exclude the presence of any bubbles, and frequent checks were made during the course of an experiment. Nevertheless the possibility that a very small gas bubble might have been present on some occasions cannot be excluded. By injecting air from an Agla micrometer syringe (Burroughs Wellcome and Co., London) into the closed chamber it was found that a bubble of 2 μl. was readily noted and was considerably larger than any bubble that could have escaped detection during an experiment. Hence it can be safely stated that an error no larger than 1 % could have been introduced into the oxygen measurement by the presence of undetected gas bubbles in the chamber.

The errors in the measurement of short-circuit current, though difficult to assess, cannot be more than ± 5 %. This figure is supported by the results in Table 1, if equality of net sodium flux and short-circuit current is accepted and the variations of the results are assigned equally to experimental error in the isotope results and in the short-circuit current measurements.

From the above considerations the coefficient of variation for a single determination of the ratio of ions transported to molecules of oxygen consumed would be approximately ± 9 %, with the error in the mean value of the 120 measurements being less than ± 1 %. The ratio might have been overestimated by an additional maximal error of 6 % because of the possible dead-space and gas-bubble error discussed above.

Effect of posterior-pituitary preparations. Ussing & Zerahn (1951) have shown that extracts of mammalian posterior-pituitary glands increase active sodium transport and short-circuit current through the isolated frog skin. The effects of such extracts on oxygen consumption and short-circuit current are shown in Table 2. Only the experimental periods just before and just after addition of the neurohypophyseal preparation to the solution bathing the inside of the skin are presented here. Not more than 30 min. elapsed between these two consecutive periods. In all instances neurohypophyseal extract resulted in the expected increase in ion transport and in all but one experiment with the whale powder a simultaneous increase in oxygen consumption was observed. This one exceptional experiment also showed only a small effect of the hormone preparation on ion transport. The increases in both oxygen consumption and sodium transport are highly significant ($P < 0{\cdot}001$, t test). If the one exceptional experiment as well as the very high value obtained in Expt. 17, Table 2, are omitted, the ratio of the increment in sodium transport to the increment of oxygen consumption yields a mean value of 18·2 sodium ions transported/molecule of oxygen consumed, with a standard error of ± 2·3.

A complete analysis of variance of the results for the two periods before and the two periods after exposure to neurohypophyseal hormones indicate that there was no significant change in oxygen consumption by the skin with time. However, to eliminate completely this possibility, as well as to provide a check by an independent method, a series of measurements were made of oxygen consumption by conventional manometric techniques in a Warburg apparatus. Paired observations with and without the addition of Pitressin were made on the two halves of the ventral-abdominal skin of 15 frogs. As observations were made for two periods of 1 hr. on each half-skin, 30 simultaneous paired comparisons were obtained. In every instance the half-skin to which Pitressin had been added showed a higher oxygen consumption/mg. dry wt. than its control half. The mean increase was 23 % (Table 3).

A comparison of the polarographic and manometric measurements of the effect of neurohypophyseal preparations on oxygen consumption by the isolated frog skin is given in Table 3. Results are expressed as Q_{O_2} (μl. of O_2 consumed/mg. dry wt.). The polarographic data refer only to the period

before and that immediately following addition of the neurohypophyseal preparation; the results include those with Pitressin, whale posterior-pituitary powder and pure vasopressin and oxytocin. The manometric results were obtained only with Pitressin. The slightly higher rates of oxygen consumption both before and after addition of neurohypophyseal preparations found with the polarographic method are probably the result of slight stretching and perhaps of short-circuiting the skin during the polarographic measurements. By both methods, however, a significant and similar increase in oxygen consumption resulted from the neurohypophyseal preparations. The higher Q_{O_2} values obtained by the polarographic method support the view that this method does not underestimate oxygen consumption and thereby give falsely high ratios of ion transport to oxygen consumption.

Table 2. *Effect of neurohypophyseal preparations on oxygen consumption and ion transport*

Expt.	Oxygen consumption μmole/cm.^{2}/hr. Before	After	ΔO_2	Sodium transport μequiv./cm.^{2}/hr. Before	After	ΔNa	$\Delta Na/\Delta O_2$
			Effects of Pitressin				
1	0·331	0·447	0·116	2·51	3·22	0·71	6·12
2	0·454	0·535	0·081	1·50	3·18	1·68	20·7
3	0·291	0·386	0·095	1·29	2·45	1·16	12·2
4	0·226	0·318	0·092	1·43	2·40	0·97	10·5
5	0·326	0·386	0·060	2·26	3·76	1·50	25·0
6	0·250	0·304	0·054	1·34	2·55	1·21	22·4
7	0·290	0·405	0·115	1·56	2·58	1·02	8·9
8	0·308	0·336	0·028	2·55	3·54	0·99	35·4
9	0·429	0·486	0·057	2·16	3·80	1·64	28·8
10	0·153	0·180	0·027	0·64	1·29	0·65	24·1
			Effects of whale posterior-pituitary powder				
11	0·199	0·142	−0·057	0·96	1·46	0·50	—
12	0·131	0·267	0·136	1·24	2·09	0·85	6·3
13	0·248	0·284	0·036	1·68	2·74	1·06	29·4
14	0·350	0·468	0·118	2·61	3·73	1·12	9·5
15	0·218	0·250	0·032	1·90	3·28	1·38	43·1
16	0·244	0·262	0·018	1·43	2·04	0·61	33·9
			Effects of pure oxytocin				
17	0·233	0·238	0·005	0·98	1·75	0·77	154
18	0·215	0·253	0·038	1·32	1·54	0·22	5·8
19	0·270	0·381	0·111	1·56	2·68	1·12	10·1
20	0·261	0·314	0·053	1·61	2·14	0·53	10·0
21	0·242	0·309	0·067	1·70	2·57	0·87	13·0
22	0·302	0·336	0·034	1·78	2·34	0·56	16·5
			Effects of pure vasopressin				
23	0·234	0·304	0·070	1·51	2·34	0·83	13·0
24	0·270	0·314	0·044	1·67	2·38	0·71	16·1
Means	0·270	0·329	0·060	1·63	2·58	0·94	18·22
S.E.M.	—	—	±0·0089	—	—	±0·075	±2·3
P	<0·001			<0·001			

Table 3. *Comparison of polarographic and manometric measurements of oxygen consumption by frog skin*

	No. of expts.	Mean temp. (°)	Q_{O_2} Before neurohypophyseal preparation	Q_{O_2} After neurohypophyseal preparation	Mean diff.	S.E.M. diff.	*P*
Polarographic	24	25·6	1·42	1·72	0·30	0·050	<0·001
Manometric	15	25·3	1·00	1·23	0·23	0·030	<0·001

Increased O_2 consumption after addition of neurohypophyseal preparation: 21 % by polarographic measurement; 23 % by manometric measurement.

DISCUSSION

Previous workers have evaluated the ratio of the number of ions transported by various tissues per molecule of oxygen consumed by that tissue. Robertson & Wilkins (1948*b*) studied the relationship between the increase in oxygen consumption of well-washed plant tissues when placed in salt solutions and the salt accumulation by the tissue. They interpreted their results to be consistent with the 'rédox' hypothesis of Lundegårdh (1939, 1940) for active accumulation of anions by plants, although occasional ratios above 4·0 were reported.

Crane & Davies (1948, 1951) compared the hydrochloric acid production of the isolated frog gastric mucosa and the increase of oxygen consumption of that tissue after addition of histamine to the medium. Ratios for moles of hydrochloric acid formed to moles increase of oxygen uptake as high as 11·5 were obtained in individual experiments. On the assumption that the increase in the rate of respiration of the mucosa after histamine provides the energy for acid secretion, very high thermodynamic efficiencies were calculated for the secretory process. When the rate of acid production was compared, however, with the total oxygen consumption by the tissue, ratios were found lower than the average figure of 2·2 obtained by Teorell (1949). So long as it is unproven that the resting respiration of the whole mucosa cannot become available in part for the secretory process when histamine is added, these findings are not conclusive evidence for the existence of ratios above 4·0 in a tissue in which the ratio of ion transport to total oxygen consumption is as low as 2·0. This objection has been countered with the argument that the acid-secreting cells comprise only about 5 % by dry weight of the gastric mucosa (Crane & Davies, 1951), so that the ratios of acid formation to oxygen consumption by the cells actually involved in acid secretion must considerably exceed the ratio obtained for the total mucosa.

Davenport (1952) and Davenport & Chavre (1953) compared the rates of acid secretion and oxygen consumption of frog gastric mucosa and of mouse stomachs and showed that the efficiency of acid secretion, i.e. the slope of the regression line of moles of acid produced to moles of oxygen consumed, was actually variable. He concludes from his studies on frog and mouse gastric mucosae: 'we have never found the molar ratio of acid secreted to oxygen consumed to be significantly greater than 4'.

The other attempts to determine this ratio have been made on the frog skin. Lund & Stapp (1947) compared the rate of flow of current through the partially short-circuited skin with the number of electrons made available by the oxygen consumption of the skin. This ratio of output of electricity in coulombs to the coulomb equivalents of total oxygen consumption expressed as a percentage gave values close to 100 % in a few experiments (equivalent to slightly less than 4·0 for the ratio as calculated in this paper). They realized that their figures represented minimal values for this ratio because the current measured was dependent upon the external resistance of the circuit used. This experimental difficulty became surmountable by the introduction of the completely short-circuited skin preparation (Ussing & Zerahn, 1951) used in the present study.

In 1952 Linderholm made calculations of this 'coulomb' efficiency for the short-circuited skin and stated: 'the quantity of electricity transferred with the sodium ions in the active transport is found to be 70 to 90 per cent of the quantity of electricity transferred by electrons in the oxidation processes'. Linderholm's data have been restated by Conway (1954, 1955) to give a ratio of 3·28 sodium ions transported per molecule of oxygen consumed. Conway (1954, 1955) emphasizes these results to support his 'redox pump' theory (Conway, 1951). However, Linderholm's results are open to criticism on two points. His calculations were made from mean values for oxygen consumption and for ion transport in separate experiments rather than from simultaneous measurements of both values in the same skin. Of greater significance is the fact that he apparently worked with skins of very low short-circuit current. Thus his mean figure for the short-circuit current in 10 skins was 19 μA/cm.^{2} or 0·71 μequiv./cm.^{2}/hr. This is to be compared with mean short-circuit currents of 1·63 and 2·58 μequiv./cm.^{2}/hr. (Table 2, before and after neurohypophyseal preparations respectively) in the present study. Though the corresponding mean oxygen consumption of 0·27 and 0·33 μmole/cm.^{2}/hr. in the present study was greater than the mean figure of 4·8 μl./cm.^{2}/hr. or 0·21 μmole/cm.^{2}/hr. found by Linderholm, the ion transport was so much greater as to yield the high ratios reported here. Every attempt was made in the present study to secure lively, healthy young frogs which would yield high short-circuit currents. That our measurements of ion transport are not excessively high is seen in the finding by others of similar rates of ion transport by isolated frog skins (Ussing & Zerahn, 1951; Fuhrman 1952; Ussing, 1954). In frog skins showing such low rates of electrical activity as those studied by Linderholm it may be expected that the residual oxygen consumption not related to ion transport becomes a more sizeable fraction of the total oxygen consumption of the skin, thereby depressing the ratio of ion transport to oxygen consumption. In the present study all low values for this ratio were obtained from skins showing low rates of ion transport. A further indication of increasing efficiency of

ion transport with high rates of ion transport is seen from the effects of neurohypophyseal preparations. Whereas these preparations stimulated an increase in both ion transport and oxygen consumption the former was affected relatively more than the latter so that a significant increase in the ratio of ion transport to oxygen consumption was obtained. This ratio had a mean value of 6·29 before and 8·00 after addition of neurohypophyseal preparations, giving a mean difference of 1·71 with a standard error of the mean difference of $\pm 0{\cdot}32$ and $P < 0{\cdot}001$.

The present results indicate that even when compared with the total oxygen consumption of the skin the number of ions transported per molecule of oxygen consumed significantly exceeds 4·0. A similar result with the same tissue has recently been obtained by Zerahn (1956). As the frog skin contains many cells in addition to the basal-epidermal cells thought to be the site of the active ion transport (Ussing, 1949) it would seem probable that the true ratio of ion transport to oxygen consumed by cells involved in the transport would be considerably higher than the values reported here. An attempt to obtain this true ratio was made by comparing the increase of ion transport after addition of neurohypophyseal preparations with the concomitant increment of oxygen consumption. Table 2 shows the ratios so calculated ($\Delta Na/\Delta O_2$). The experimental error propagated into the calculation of these ratios is, of course, considerable; the standard deviation for a single ratio being about 50 % of the ratio. In addition, the calculation of such a ratio may be open to the same sort of criticism that Davenport has made of this type of calculation for the gastric mucosa. Nevertheless, the mean value of 18 probably gives the best estimate possible at present of the actual stoicheiometric relationship which exists between ion transport and metabolism in the fraction of cells of the tissue which are engaged in the transport process.

Davies & Ogston (1950) have modified the 'redox' hypothesis of ion transport to yield maximum ratios of 12 for the number of univalent ions transported to molecules of oxygen consumed (their mechanism II). More recently Davies (1956) has proposed a scheme consistent with even higher ratios of ion transport to tissue respiration. The present study suggests that an upper limit of 12 may be actually too low for this tissue, but a definite statement cannot be made from these results.

Though the interpretation of a ratio calculated from the increment of ion transport and oxygen consumption stimulated by neurohypophyseal preparations is of questionable validity, the results clearly indicate that ion transport and oxygen consumption are both increased in this tissue by some factor or factors contained in posterior-pituitary gland of mammalian origin. Both pure oxytocin and pure vasopressin in the large amounts used (1 unit) were found to stimulate ion transport and oxygen consumption. No attempt, however, has been made to evaluate the minimal amount of each compound which is effective. The stimulation of ion transport by crude posterior-pituitary preparations has previously been established (Ussing & Zerahn, 1951) but the effect on oxygen consumption constitutes a new finding.

No inconsistencies are encountered when the energy requirements for the high rates of ion transport are related to the energy available from aerobic metabolism in this tissue. The total energy production of the skin before and after the addition of neurohypophyseal preparations may be calculated from the mean oxygen consumption figures of Table 2, and the factor 0·116 calories/μmole of oxygen consumed (Davies & Ogston, 1950). The minimal energy requirement for sodium transport is obtainable from the mean current figures of Table 2 and an approximate value of the potential of the sodium pump calculated from the sodium influx and outflux figures of Table 1, according to Ussing & Zerahn (1951). Although the calculations utilize results from different experiments, they yield an order of magnitude for the minimal energy requirement for the ion transport through the short-circuited frog skin. This was calculated to be 8 and 10 % of total metabolism before and after neurohypophyseal preparations respectively, and 22 % for the increment of ion transport and oxygen consumption stimulated by the hormonal preparation.

The results of this study clearly indicate that the unmodified 'redox' hypothesis of ion transport in which molecular oxygen is the final electron acceptor is insufficient to account for the observations reported here on the short-circuited frog skin. It would seem premature to attempt to generalize the results obtained in this tissue to the problem of ion transport by other cells or tissues.

SUMMARY

1. Simultaneous measurements of active ion transport and oxygen consumption have been made on the isolated frog skin. The short-circuit current was used as the measure of ion transport (Ussing & Zerahn, 1951) and oxygen consumption was measured polarographically with vibrating platinum micro-electrodes.

2. The ratio of the number of sodium ions transported per molecule of oxygen consumed was found to range from 2 to 13 (120 periods of measurement) with a mean of 6·82 and a standard error of the mean of $\pm 0{\cdot}61$. In all but five periods the value of this ratio was above 4·0.

3. These results invalidate for frog skin the 'redox' hypothesis of active ion transport as formulated by Conway (1953, 1954, 1955) and Lundegårdh (1954) according to which no more than four univalent ions can be transported per molecule of oxygen consumed.

4. Crude neurohypophyseal preparations which are known to augment the active transport of sodium by the isolated frog skin are shown to increase oxygen uptake by about 20 %. Both pure oxytocin and pure vasopressin similarly increase ion transport and oxygen consumption by this tissue.

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The Anaerobic Active Ion Transport by Isolated Frog Skin

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Francis & Pumphrey (1933) and Francis (1934) found that maintenance of a membrane potential by isolated frog skin was dependent upon the presence of oxygen. Largely as a result of these early studies, as well as from the use of inhibitors of metabolism, it has become accepted that active ion transport by the isolated frog skin is entirely dependent upon oxidative sources of energy. The present results demonstrate that some active ion transport does, in fact, continue under completely anaerobic conditions though at a slower rate (about 20–40 %) than in the presence of oxygen.

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METHODS

Experiments were done on the ventral skin of 15 male frogs (*Rana temporaria*) during the late spring months. The frogs were stored and prepared as previously described (Leaf & Renshaw, 1957).

Active ion transport was measured by the short-circuit technique of Ussing & Zerahn (1951) with current readings made at intervals of 2·5 min. during the experiments. The short-circuit current was measured with a Weston d.c. milliammeter, model 622 (Weston Electrical Instrument Corp., Newark, New Jersey, U.S.A.), having full-scale deflexion of 100 μA at full sensitivity and an accuracy of 0·5% of full-scale reading. Measurements of ionic fluxes have not yet been done under anaerobic conditions to validate the equality of short-circuit current and active sodium transport that applies aerobically (Ussing & Zerahn, 1951). The principle that short-circuit current represents active ion transport must, however, apply although it possibly measures something less than the total active ion transport engaged in by the skin under these conditions.

The skins were mounted in a chamber similar to that described by Ussing & Zerahn (1951) and gassed through the bubbling and mixing device they describe. Volumes of 10·0 or 12·0 ml. of frog Ringer solution (112 mM-NaCl, 1·88 mM-KCl, 2·38 mM-$NaHCO_3$ and 0·89 mM-$CaCl_2$) were used as the bathing medium on each side of the skin and samples were removed as desired by pipette. No substrate was added to the medium but 0·1 mg. of calcium streptomycin and 165 units of sodium penicillin were contained in each ml. The medium was equilibrated with room air, 'oxygen-free' N_2 (British Oxygen Co.) or 100% CO (Imperial Chemical Industries, Ltd., guaranteed to contain less than 0·1% of O_2). The N_2 and CO were further deoxygenated by bubbling through a double train of vanadyl sulphate solution as recommended by Meites & Meites (1948). The O_2 tension in the medium bathing each side of the skin was continuously recorded by two vibrating Pt micro-electrodes assembled and operated as previously described (Leaf & Renshaw, 1957).

The effect of neurohypophyseal hormones was tested by addition of 2 units (0·10 ml.) of Pitressin (Parke, Davis and Co. Ltd., Batch no. LS588F) to the medium bathing the inside of the skin.

Lactic acid production by the skins was measured in the bathing medium during five of the short-circuit current experiments and in four additional experiments on isolated skins in which the ventral skin of each animal was divided in half. One half was incubated in 5·0 ml. of frog Ringer anaerobically with N_2 for 1 hr. while the other half was similarly incubated with room air or 100% O_2. In the latter experiments the medium and the skins were separately analysed for lactic acid. The skins were dropped into liquid air, pulverized in a chilled mortar and extracted with 1–2 ml. of cold 10% trichloroacetic acid. Lactic acid determinations were made on this extract and washings of the residue. Lactic acid was measured by the method of Barker & Summerson (1941). Dry weight of skins was obtained after drying overnight at 105°.

RESULTS

Fig. 1 shows the characteristic pattern of ion transport by the isolated frog skin when exposed to anaerobic conditions. The short-circuit current drops rapidly, as oxygen is removed, to a level 20–50% of that in the presence of oxygen and becomes quite stable at this lower level for 1–2 hr. On re-exposure to air there is a rapid return toward the pre-anaerobic levels of ion transport. If the anaerobiosis is prolonged much beyond this period of time the ion transport is no longer returned by oxygen to control rates; the skin has suffered some irreversible change from protracted anoxia.

As shown in Fig. 1, changing from pure nitrogen to pure carbon monoxide has no further effect on short-circuit current. In none of the nine experiments in which carbon monoxide was substituted for the nitrogen during anaerobic incubation was there any additional effect attributable to it.

The application of neurohypophyseal hormones to the skin was found to have no effect during anaerobic incubation in eight experiments as shown in Fig. 1, and had only a questionable and slight effect in two other experiments in which it was tested. This is in contrast to the prompt and large stimulation of ion transport it characteristically produces under aerobic conditions (Ussing & Zerahn, 1951).

To learn more about the sources of energy for the skin during anaerobiosis, estimations of lactate were made on the fluid bathing both sides of the skin in five experiments. Table 1 shows the lactate accumulation on the inside and outside of the skins during aerobic and anaerobic incubation as well as the simultaneous anaerobic ion transport. Results are expressed as μmole/3·14 cm.^{2} of skin and the individual dry weights of this area of skin are recorded. It is evident that lactate is formed both

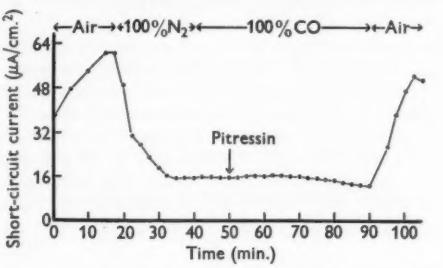

Fig. 1. A comparison of aerobic and anaerobic active ion transport by the isolated frog skin. The rate of active ion transport is indicated on the ordinate as the short-circuit current in μA/cm.^{2} of skin. The actual area of skin used was 3·14 cm.^{2}. Time is the abscissa. After an initial period of aeration, the medium bathing the skin was equilibrated with 100% nitrogen. At 23 min. the polarographic tracing indicated that the medium was 'oxygen free'. The rate of ion transport dropped rapidly initially in the absence of oxygen but then became stable. Altering the gas phase to 100% carbon monoxide produced no further effect. Addition of 2 units of Pitressin to the medium bathing the inside of the skin was without effect on ion transport anaerobically. Re-aeration after 70 min. without oxygen returned the rate of ion transport toward control levels.

Table 1. *Lactic acid formation and anaerobic ion transport by isolated frog skin*

The area of skin was 3·14 cm.². Inside and outside refer to medium bathing inside and outside of frog skin.

Expt. no.	Time (min.)	Lactic acid (μmoles) Outside	Lactic acid (μmoles) Inside	Time (min.)	Lactic acid (μmoles) Outside	Lactic acid (μmoles) Inside	Anaerobic ion transport (μequiv.)	Dry wt. (mg.)
1	25	0	0·10	73	0	0·24	1·67	15·0
2	28	0·08	0·37	75	0·06	0·11	1·47	17·5
3	25	0·03	0·15	47	0·02	0·38	1·55	17·4
4	53	0·05	0·10	55	0·04	0·14	1·05	19·5
5	29	0·06	0·15	60	0·04	0·10	0·97	12·2

Table 2. *Aerobic and anaerobic lactic acid formation by isolated frog skin*

Expt.	Lactic acid (μmole/100 mg. of wet skin/hr.) In air	In O_2	In N_2
1	0·05	—	0·42
2	0·10	—	0·33
3	—	0·20	0·30
4	—	0·22	0·41

aerobically and anaerobically and that under both conditions it accumulates more in the medium bathing the inside of the skin.

To test the possibility that the formation of lactate aerobically was due to insufficient aeration of the skin when in the short-circuit current chamber, four additional experiments were done in test tubes fitted with a bubbling device so that one half of a skin could be gassed with pure nitrogen and the other half with air or 100 % oxygen. Table 2 shows that, even in 100 % oxygen, lactate is formed by this tissue, but that anaerobically the rate of lactate formation is greater than aerobically. The anaerobic rate was up to eight times higher than the aerobic rate. Results are expressed as μmoles of lactic acid/100 mg. wet wt. of skin/hr. The quantities of lactic acid found in the skin were small compared with that in the medium and the values presented are the totals for skin and medium.

DISCUSSION

Francis (1934) studied the effect of oxygen removal on the membrane potential of isolated frog skin and stated that: 'If the rate of respiration falls off there is no reserve material or auxiliary process by which the potential can be maintained in spite of oxygen lack.' Lund & Stapp (1947) noted, however, that a current could be drawn for several hours from the partially short-circuited frog skin under presumably anaerobic conditions, but they made no comment regarding this finding.

The present study emphasizes that the inhibition of active ion transport during oxygen lack is only partial, and for a time at least, is reversible. The results show that ion transport can continue anaerobically; they are compatible with the view that anaerobic glycolysis can supply the required energy for this active process. This finding possibly links the mechanism of ion transport in human red blood cells, which are known to be dependent upon glycolytic supplies of energy (Danowski, 1941; Harris, 1941; Maizels, 1951) with the study of ion transport by other tissues in which aerobic-energy sources have been thought to be essential for active transport. It is likely that a requirement for the ion-transporting mechanism is a supply of adenosine triphosphate, and it makes no difference whether this is provided by aerobic or anaerobic metabolism.

The lack of demonstrable anaerobic ion transport by some other tissues which have been tested (Terner, Eggleston & Krebs, 1950; Mudge, 1951*a*, *b*; Whittam & Davies, 1953) is probably dependent upon at least two factors: (1) the insufficient supply of energy anaerobically by some tissues, notably kidney and brain; (2) the insensitivity of most methods of measuring active ion transport, which do not permit a distinction between a marked degree and complete suppression of active transport.

The view that ion transport was specifically dependent upon aerobic sources of energy has given rise to the oxidation–reduction hypothesis of active transport in which the transference of electrons from substrate to molecular oxygen via the oxidation–reduction reactions of the cytochrome system is considered to be the essential feature of the transporting mechanism. Evidence has been recently provided that the oxidation–reduction hypothesis is insufficient to account for observed rates of ion transport by frog skin (Zerahn, 1956; Leaf & Renshaw, 1957). The continued transport by this tissue in the absence of oxygen, and even in the presence of 100 % carbon monoxide, provide an even more forceful demonstration that active ion transport is not dependent upon oxidations involving molecular oxygen. Although the initial decrease in ion transport rate that occurs when oxygen is removed from the skin could be the result of cessation of electron transport through the

cytochrome system, a second ion-transporting mechanism would then have to be postulated to explain the anaerobic transport. It seems simpler at present, therefore, to assume that the higher rates of ion transport that occur aerobically than anaerobically are due to the more efficient energy-producing reactions in aerobic than in anaerobic metabolism, and that high-energy phosphate compounds produced by either anaerobic or aerobic pathways of metabolism are utilizable by a single ion-transporting mechanism which, for a given ion, may be similar in different tissues.

From the figures in Table 1 for active ion transport and simultaneous anaerobic lactate formation one can calculate the number of univalent ions transported per molecule of lactic acid formed. Values of 3·9–8·6 are obtained, which would mean some two to six univalent ions transported per high-energy phosphate bond synthesized, assuming glycogen to be the substrate metabolized. However, this calculation is tentative, since it is based on only a few measurements and since the total lactate production of this complex tissue must be used rather than just that part of it which supplies energy for the ion-transport process.

It has been shown that the increased ion transport produced aerobically in this tissue by neurohypophyseal hormones is associated with an increased oxygen uptake (Leaf & Renshaw, 1957). The absence of an effect of these hormones anaerobically in this tissue suggests that the action of the hormones on ion transport is somehow associated with their ability to increase the availability of aerobic-energy sources for operation of the ion-transport mechanism.

SUMMARY

1. Active ion transport by the isolated frog skin has been measured electrically by the method of Ussing & Zerahn (1951) under aerobic and anaerobic conditions.

2. In the presence of 100% nitrogen or 100% carbon monoxide ion transport was found to continue for over an hour, although at a lower rate (about 20–40%) than in the presence of oxygen.

3. Under anaerobic conditions an increase in lactic acid formation was demonstrated which is compatible with the view that the energy for active ion transport anaerobically is supplied by glycolysis. In five experiments values were obtained indicating that two to six univalent ions were transported per high-energy phosphate bond synthesized anaerobically.

4. The stimulatory effect of neurohypophyseal hormones on ion transport in this tissue is absent during anaerobiosis.

5. The results conflict with the oxidation–reduction hypothesis of ion transport in which molecular oxygen is the final electron acceptor and support the view that the ion transport in this tissue is dependent upon high-energy phosphate compounds which can be supplied by either aerobic or anaerobic metabolic pathways.

One of us (A.L.) wishes to express his gratitude to Professor H. A. Krebs for the privilege of working in his laboratory and for helpful criticism, and to Professor Walter Bauer for encouragement and support. We would like to thank Dr Henry Harris for helpful suggestions.

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Studies on the Biosynthesis of Cholesterol

4. DEGRADATION OF RINGS C AND D*

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Chemical degradation of cholesterol biosynthesized from labelled acetic acid has been the subject of several communications from our own and other Laboratories [for review and bibliography see Cornforth (1954)]; the aim has been to discover which carbon atoms in cholesterol originate from the methyl group of acetic acid and which from the carboxyl group. The completion of this project is described in the present paper. When work was begun, the biosynthetic origin of C-7, C-8, C-9, C-11, C-12, C-14, C-15, C-16 and C-17 was unknown, and confirmation was desirable for C-13 and C-18. Later, the derivation of C-7 from the methyl group of acetate was proved by Bloch (1953) and by Dauben & Takemura (1953).

METHODS AND EXPERIMENTAL

Biosynthesis

[^{14}C]*Cholesterol.* Labelled cholesterol was obtained by incubation of liver slices of young rats with either [*Me*-^{14}C]acetate or [*carboxy*-^{14}C]acetate as described previously (Cornforth, Hunter & Popjak, 1953*a*). The unsaponifiable material extracted from the alkaline digest of the slices was first chromatographed on alumina (B.D.H. chromatographic grade) previously washed with methyl formate. Light petroleum (b.p. 40–60°), acetone–ethyl ether (1:1, v/v) and methanol were used for elution in succession. Digitonides were prepared from the acetone–ether eluate. The cholesterol, after decomposition of the digitonide by pyridine–ether, was purified through the dibromide and recrystallized from methanol. After preliminary dilution with inactive material, the cholesterol samples thus prepared were finally obtained for degradation as the acetates. Small samples of each were set aside as standards for assay for ^{14}C.

Derivatives of [^{14}C]cholesterol biosynthesized from $CH_3.{}^{14}CO_2H$ and $^{14}CH_3.CO_2H$ will be referred to as carboxyl-labelled and methyl-labelled respectively. Carboxyl-labelled cholesteryl acetate (2·324 g.) with a specific activity of 3·58 μc/g. and methyl-labelled cholesteryl acetate (2·538 g.) with a specific activity of 5·28 μc/g. served as starting materials for all degradations.

It might be mentioned that the [^{14}C]squalene biosynthesized from [*Me*-^{14}C]acetate, the complete degradation of which has been reported previously (Cornforth & Popjak, 1954), was obtained from these same biosynthetic experiments.

Manipulations before fission

In order to separate from each other the carbon atoms in ring D of cholesterol (I; R = H) it was necessary to open this ring. Our first task, achieved by modification of procedures already known, was the replacement of the existing double bond at 5:6 by a double bond at 14:15.

Bromination of cholesteryl esters with *N*-bromosuccinimide and dehydrobromination of the crude product containing 7-bromo compounds is an important method for preparing cholesta-5:7-dienes and several procedures have been published; we followed that of Bernstein, Binovi, Dorfman, Sax & Subbarow (1949). The isomeric 4:6-diene is always formed as a by-product of this reaction, and to secure a good recovery of pure 5:7-diene (II; R = Ac) would have been difficult. To the partially purified product from the [^{14}C]cholesterols, after saponification, a relatively large quantity of inactive cholesta-5:7-diene-3β-ol (II; R = H) was therefore added; radioactive diene (II; R = H) sufficiently pure for the next step was then obtained without trouble.

Partial hydrogenation of the diene to cholest-7-en-3β-ol (III; R = H) proceeded smoothly in benzene solution with Raney nickel as catalyst (Ruyle *et al.* 1952). Migration of the 7:8 double bond to the 8:14-position occurred on shaking III (R = H) with hydrogen and a platinum catalyst in acetic acid (cf. Wieland, Rath & Benend, 1941); the product, cholest-8(14)-en-3β-ol (IV; R = H) was formed almost quantitatively.

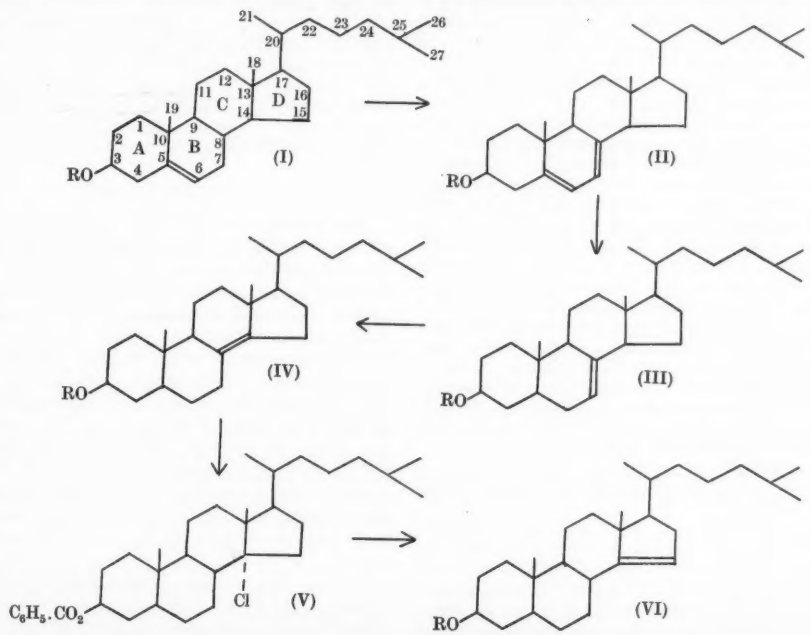
The free hydroxyl group in IV (R = H) was benzoylated before the next isomerization, as this facilitated isolation of the product. The original procedure of Schenck, Buchholz & Wiese (1936) for

* Part 3: Cornforth & Popjak (1954).

isomerization of this benzoate [IV; R = C_6H_5.CO] with hydrogen chloride in chloroform at 0° gave moderate yields even when more concentrated solutions were used (Heilbron & Wilkinson, 1932) and a milder method was sought. Addition of hydrogen chloride took place in chloroform solution at −30° and the adduct, presumably the 14α-chloro derivative (V), could if necessary be isolated. When the chloroform solution containing this adduct was shaken with aqueous $NaHCO_3$ dehydrochlorination occurred slowly, but it was accelerated on addition of ether. Cholest-14-en-3β-yl benzoate (VI; R = C_6H_5.CO) was then obtained in 75–80% yield. By renewed treatment of the mother liquor containing the 8(14)-isomeride the yield was raised to 90%.

Radioactive cholesta-5:7-dien-3β-ols. To carboxyl-labelled cholesteryl acetate (2·267 g.) in light petroleum [27·5 ml.; b.p. 66–67°; purified according to Bernstein *et al.* (1949)] was added finely powdered *N*-bromosuccinimide (1·152 g.) and the mixture was boiled under reflux for 12 min. in the light and heat from two Photoflood lamps (Royal Ediswan no. 1). Collidine (1·14 ml.; redistilled and kept over KOH) was added immediately before the lamps were switched off. Succinimide was removed by filtration and the solvent was evaporated at room temperature under reduced pressure. To the residue one half of a solution of collidine (0·46 ml.) in xylene (13·6 ml.) was added; evacuation was then resumed for a short time before adding the remainder. The mixture was boiled under N_2 at atmospheric pressure for 15 min. Collidine hydrobromide was removed by washing with water; the xylene solution was extracted successively with 70 ml. portions of 0·5 N-HCl, water, 0·5 M-$NaHCO_3$ and water. It was then dried ($MgSO_4$) and stirred with a little charcoal, filtered, and evaporated at low pressure (capillary fed with N_2). After removal of the remaining xylene in high vacuum the residue was dissolved in acetone–methanol (1:2, v/v; 7·5 ml.), seeded and left under N_2 at −5°. The solid was collected at −5° and washed with small amounts of 1:2 (v/v) and then 1:5 (v/v) acetone–methanol; it weighed 1·57 g. This material was boiled under N_2 for 45 min. with 16 ml. of methanolic KOH (5%, w/v). Ether was added and the mixture was washed repeatedly with small quantities of ice-water. Non-radioactive cholesta-5:7-dien-3β-ol (7·5 g.) dissolved in ether was then added and the solution was concentrated under N_2 to 30–40 ml. When crystals appeared, warm methanol was added; the mixture was cooled to −5° and the solid was collected after 2 hr., washed with chilled methanol, and dried *in vacuo*. The yield was 7·90 g.; m.p. 142–145° (melting-points, except those taken on the Kofler block, were observed in open capillaries and are uncorrected).

Methyl-labelled cholesteryl acetate (2·524 g.) was treated in the same manner, but for some undiscovered reason the recovery of crude crystalline product was lower (0·949 g.). Since the ultraviolet absorption at 282 mμ. of the mother liquor indicated that much diene had remained in solution, the solid and the mother liquor were both saponified. To the product from the mother liquor, non-radioactive cholesta-5:7-dien-3β-ol (1·5 g.) was added. The mixture was dissolved in a minimum of boiling acetone and allowed to crystallize at −5°. The crystals after washing with chilled 2:1 (v/v)



acetone–methanol and drying weighed 1·46 g.; m.p. 136–141°. This was added along with 6 g. of non-radioactive diene to the product from saponification of the solid and the whole was recrystallized from acetone to give 6·866 g., m.p. 145–148°.

Radioactive cholest-7-en-3β-ols. To carboxyl-labelled cholesta-5:7-dien-3β-ol (7·7 g.) in benzene (200 ml.; thiophen-free; previously refluxed with Raney nickel), Raney nickel (approx. 2 ml. of a settled suspension in benzene) was added and H_2 was admitted at 50 lb./in.^{2}. After shaking for 2 hr. and leaving overnight a small amount of diene had remained unreduced, as shown by absorption at 282 mμ., and shaking was continued for 3 hr. The solution was filtered through kieselguhr and the solvent was removed under N_2 at low pressure. The residue was recrystallized from methanol (80 ml.) and dried *in vacuo*; 6·90 g., m.p. 124°. A second crop after recrystallization weighed 0·16 g.; m.p. 119–121°.

The methyl-labelled analogue was prepared in the same way from the corresponding diene (6·866 g.); yield 6·66 g., m.p. 122–123°. Optical rotations were not measured with these preparations but preliminary investigations with non-radioactive material gave a product m.p. 124°, $[\alpha]_D \pm 0°$. Schenck *et al.* (1936) gave m.p. 122–123°; $[\alpha]_D \pm 0°$. (Specific rotations refer to $CHCl_3$ solutions when the solvent is unspecified.)

Radioactive cholest-8(14)-en-3β-ols. Carboxyl-labelled cholest-7-en-3β-ol (7·06 g.) was triturated with acetic acid (80 ml. redistilled from CrO_3) and the suspension was added to Pt black (from 340 mg. of PtO_2) in acetic acid (80 ml.). The mixture was shaken for 2 hr. with H_2. Chloroform was added to dissolve the product; the catalyst was removed by filtration and the solvents by evaporation at low pressure under N_2. The residue was recrystallized from methanol to give 7·06 g., m.p. 121°. A second crop after recrystallization weighed 0·035 g.; m.p. 119°. The net gain in weight is attributable to solvation.

Methyl-labelled cholest-7-en-3β-ol (6·6 g.) was treated in the same manner to give 6·41 g., m.p. 121°.

The non-radioactive product had m.p. 120°; $[\alpha]_D^{24} + 21°$. Schenck *et al.* (1936) gave m.p. 119–120°; $[\alpha]_D^{21} + 20·36°$.

Radioactive cholest-8(14)-en-3β-yl benzoates. To carboxyl-labelled cholest-8(14)-en-3β-ol (7·095 g.) in pyridine (35 ml.) benzoyl chloride (4·7 ml.) was added with shaking. After 21 hr. the mixture was stirred with water (500 ml.) for 1 hr. and the solid was collected, washed with water and methanol, dried and recrystallized from acetone. It was later suspected that benzoylation was incomplete and the procedure was therefore repeated. This gave 5·72 g.; melting at 111–113° to a cloudy liquid which cleared at 138–140°; from crystallization of mother liquors a further 1·67 g. (m.p. 107–110°) and 0·42 g. (m.p. 108°) were obtained.

Methyl-labelled cholest-8(14)-en-3β-ol (6·4 g.) was twice benzoylated in similar fashion to give 6·04 g., m.p. 112–113° (clear at 140–141°), 0·70 g., m.p. 106–109° (clear at 133°) and 0·355 g., m.p. 112° (clear at 134°).

The non-radioactive product had m.p. 114° (clear at 144°), $[\alpha]_D^{21} + 11°$. Schenck *et al.* (1936) gave m.p. 115° (clear at 140°), $[\alpha]_D^{20} + 8·53°$.

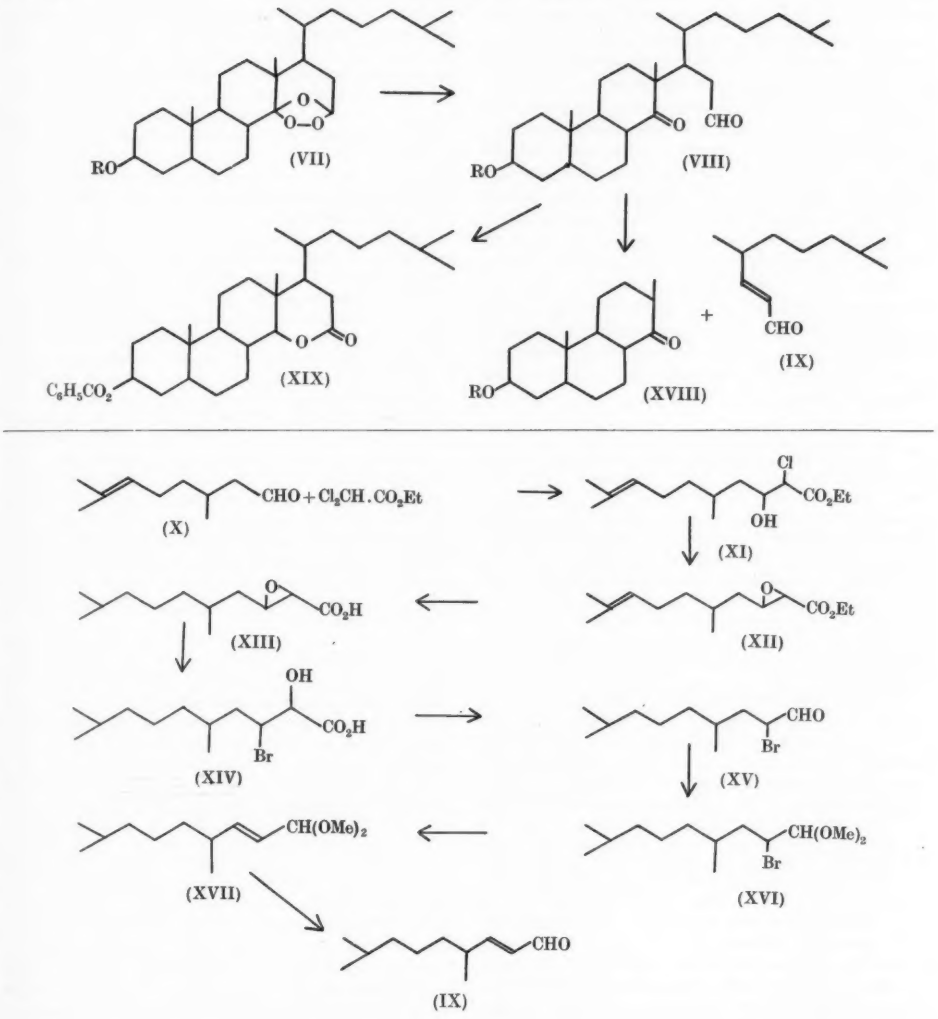
Radioactive cholest-14-en-3β-yl benzoates. Carboxyl-labelled cholest-8(14)-en-3β-yl benzoate (3·0 g.) was dissolved in $CHCl_3$ (6 ml.) and a gentle stream of dry HCl was passed for 2 hr. through the solution at −30°. The pressure in the reaction vessel was then lowered to about 20 mm. without interrupting the cooling; this procedure removed most of the HCl and some of the $CHCl_3$. The residue was poured into 0·5 M-$NaHCO_3$ (10 ml.), a little cold $CHCl_3$ being used for washing. Ether was then added; the mixture was left for ½ hr., shaken, and the aqueous layer run off. The product which had separated from solution was redissolved ($CHCl_3$) and the dried ($MgSO_4$) solution evaporated at low pressure. The residue was recrystallized as rapidly as possible from benzene (8 ml.) by addition of ethanol (16 ml.). The product (1·88 g.) crystallized in needles, m.p. 167°. A second run with 2·70 g. of the 8(14)-ene gave 1·735 g., m.p. 168·5°. The mother liquors from these two runs on further crystallization gave 0·895 g., m.p. 164·5–167°. The total from 5·72 g. was thus 4·51 g. or 79%. The less pure 8(14)-ene from the previous stage (1·67 + 0·42 g.) was also isomerized, after being united with material (0·7 g., m.p. 111°) from the mother liquors of the first isomerizations, to give 2·01 g., m.p. 167–167·5°. Finally the combined mother liquors were evaporated and the residue was isomerized: this gave 0·435 g., m.p. 166°. Thus from a total of 7·79 g. of 8(14)-ene the yield was 6·955 g. (89%) of a satisfactory product.

The methyl-labelled cholest-8(14)-en-3β-yl benzoate (6·04 + 0·70 + 0·355 g.) was isomerized in similar fashion to give a total of 6·005 g. (92%), m.p. 165°. The highest m.p. observed with non-radioactive cholest-14-en-3β-yl benzoate was 168·5°; $[\alpha]_D^{22} + 31·5°$. Schenck *et al.* (1936) gave m.p. 168°; $[\alpha]_D^{22} + 32·54°$.

14α(?)-Chlorocholestan-3β-yl benzoate. Non-radioactive cholest-8(14)-enyl benzoate (1 g.) in $CHCl_3$ (3 ml.) was treated at −30° with HCl as above; after removal of excess of HCl the remaining solvent was evaporated at −30° in high vacuum. To the solid white residue was added light petroleum (b.p. 40–60°) cooled to −30°. The solid was collected quickly on a filter cooled by solid CO_2 and dried at −30° in high vacuum. The product after being kept overnight at −5° had m.p. 153–156° (clear at 158°). (Found: Cl, 6·7. $C_{34}H_{51}O_2Cl$ requires Cl, 6·7%.) This substance (200 mg.) dissolved in ether (5 ml.) and $CHCl_3$ (2 ml.) was shaken with 0·5 M-$NaHCO_3$. The product on crystallization from benzene–ethanol gave cholest-14-en-3β-yl benzoate (0·13 g.), m.p. 166–166·5°.

Cleavage of ring D

The radioactive material was now ready for cleavage of ring D. Ozonolysis of the 14-ene (VI), reduction of the ozonide (VII) and pyrolysis of the resulting keto aldehyde (VIII) was the plan, for this sequence had been demonstrated with the ergostene analogues (Achtermann, 1934; Laucht, 1935). Cholest-14-en-3β-ol (VI; R = H), prepared by saponification of the benzoate (Schenck *et al.* 1936), was ozonized in hexane and the ozonide was reduced by zinc and acetic acid. After several trials we chose to pyrolyse the non-crystalline keto aldehyde (VIII) in *cyclo*hexane at 210–220°, having observed the appearance under these conditions of a substance volatile in steam and absorbing maximally at 222 mμ. These properties would be expected of the aldehyde (IX). A crystalline semicarbazone was in fact prepared from the steam-volatile material, and its composition indicated its origin from a C_{11} carbonyl compound.



The semicarbazone was optically active. Thereby, its synthesis from a product of known configuration—already desirable in order to prove its structure and to secure more material for experimental degradations—offered the opportunity of determining the absolute configuration of cholesterol. We therefore proceeded to synthesize the semicarbazone of the aldehyde (IX) from (+)-citronellal. The identity of the two specimens from cholesterol and from citronellal, and the stereochemical conclusions drawn from this identity, have already been reported (Cornforth, Youhotsky & Popjak, 1954); experimental details of the synthesis are now given. The transformation

$$R.CH_2.CHO \rightarrow R.CH{:}CH.CHO$$

might be effected by several methods; we chose one which seemed unlikely to cause racemization at any stage.

Condensation of (+)-citronellal (X) with ethyl dichloroacetate and magnesium amalgam (method of Darzens & Levy, 1937) gave the β-hydroxy-α-chloro ester (XI) which was converted by ethanolic sodium ethoxide into the glycidic ester (XII). The

glycide ester (XII) was saponified and the acidic product was hydrogenated in ethyl acetate over a platinum catalyst. The crude saturated glycidic acid (XIII) in benzene readily absorbed hydrogen bromide. The resulting α-hydroxy-β-bromo acid (XIV) was oxidized by lead tetraacetate in benzene. A neutral product consisting principally of the bromo-aldehyde (XV) was obtained. This was immediately converted into the dimethyl acetal (XVI) by reaction with methanol containing ethyl orthoformate and a little hydrogen chloride. This bromo-acetal was distilled but not further purified; it was boiled with methanolic KOH to produce the unsaturated acetal (XVII). Gentle hydrolysis with acid then produced a substance absorbing maximally at 222 mμ. and affording a crystalline semicarbazone identical with the semicarbazone from the product of pyrolysis (IX).

Ozonization of cholest-14-en-3β-ol. Cholest-14-en-3β-ol, m.p. 130–131°, was prepared from the benzoate by the procedure of Schenck *et al.* (1936). Ozonized oxygen was passed into a solution of this alcohol (2·5 g.) in hexane (250 ml.) at 0° until a blue colour persisted. An amorphous solid was precipitated. This was collected (2·025 g.) and shaken in acetic acid (60 ml.) with Zn powder (2·5 g.) for 45 min., the temperature rising to 40°. Water (140 ml.) and ether (100 ml.) were added, the Zn was removed and the ethereal layer after washing with water and 0·5 M-$NaHCO_3$ was dried ($MgSO_4$) and evaporated at low pressure. The glassy residue containing the keto aldehyde (VIII; R = H) weighed 1·5 g.

Pyrolysis of 3β-hydroxy-14-oxo-14:15-secocholestan-15-al. The keto aldehyde (302 mg.) in *cyclo*hexane (15 ml.) was sealed under N_2 in two tubes which were heated at 210–220° for 16 hr. The tubes were washed out with *cyclo*hexane (15 ml.) and the combined solutions were distilled in steam. A portion (0·1 ml.) of the *cyclo*hexane layer, after dilution to 40 ml. with ethanol, showed maximum absorption at 222 mμ., optical density 0·425. To the *cyclo*hexane layer semicarbazide hydrochloride (50 mg.) and NaOAc,$3H_2O$ (50 mg.) dissolved in a little water were added, followed by methanol (10 ml.). The mixture was boiled vigorously for 5·5 hr., cooled and diluted with water and concentrated at low pressure. A crystalline solid separated from the aqueous layer. This was collected, washed with water and dried (35 mg.; m.p. 85–105°). Crystallization from benzene–light petroleum afforded plates, m.p. 120–122° (Kofler block). After one recrystallization from aqueous methanol and a final, slow crystallization from 50% (v/v) aqueous ethanol, large diamond-shaped plates were obtained, m.p. 133°, remelting after cooling at 124°; $[\alpha]_D^{20}$ − 24·5° (c = 1 in dioxan). (Found: C, 64·0; H, 10·6; N, 18·8. $C_{19}H_{23}ON_3$ requires C, 64·0; H, 10·2; N, 18·7%.)

Synthesis of (−)-4:8-dimethylnon-2-en-1-al semicarbazone. Magnesium (2·4 g.) was stirred vigorously with mercury (240 g.) until amalgamation was complete. (+)-Citronellal (15·4 g.; $[\alpha]_D^{20}$ + 8°) and ethyl dichloroacetate (15·7 g.) were mixed in dry ether (20 ml.) and about half of this solution was added to the stirred amalgam which had been covered with dry ether (80 ml.). A vigorous reaction soon set in and external cooling was needed to supplement an efficient reflux condenser while the remaining solution was added as rapidly as possible. When the reaction subsided the mixture was stirred for 2 hr. Ice and 2 N-H_2SO_4 were added. The ethereal layer was washed with water and with M-$NaHCO_3$, dried and evaporated. Fractional distillation of the residue gave *ethyl 2-chloro-3-hydroxy-5:9-dimethyldec-8-en-1-oate* (11·4 g.), b.p. 94–97°/0·08 mm., n_D^{20} 1·4731 (Found: C, 61·1; H, 9·3; Cl, 12·5. $C_{14}H_{25}O_3Cl$ requires C, 60·8; H, 9·05; Cl, 12·8%.) If the reaction was conducted more slowly, the yield was lower, perhaps because self-condensation of the citronellal became more important.

The ester (11·4 g.) was treated at room temperature with a slight excess of sodium ethoxide in dry ethanol. Sodium chloride separated at once. After a few minutes, sufficient acetic acid to neutralize the mixture was added. Ether and water were added and the ether-soluble product was distilled. Ethyl 2:3-epoxy-5:9-dimethyldec-8-en-1-oate (7 g.), somewhat impure, was collected at 89–91°/0·06 mm. (Found: C, 69·1; H, 10·3. $C_{16}H_{28}O_3$ requires C, 70·0; H, 10·0%.)

The glycidic ester (7 g.) was saponified by heating for 1 hr. with ethanol (31·5 ml.) and N-NaOH (31·5 ml.). The ethanol was removed at low pressure, a trace of neutral material was extracted with ether and the cold aqueous solution was carefully acidified to Congo red with 2 N-H_2SO_4 in the presence of ether. The crude glycidic acid remained as a thick oil (5·7 g.) on evaporation of the ether. This was hydrogenated in ethyl acetate (96 ml.) over PtO_2 (240 mg.). In less than 1 hr., slightly more than one equivalent of hydrogen was absorbed and reaction then ceased. On removal of catalyst and solvent, crude 2:3-epoxy-5:9-dimethyldecanoic acid (5·7 g.) remained.

This product was dissolved in benzene (75 ml.) and dry HBr was added until an excess was present, which was then removed by thorough washing with water. Evaporation of the benzene *in vacuo* then left crude 3-bromo-2-hydroxy-5:9-dimethyldecanoic acid (7·16 g.). This acid was dissolved in a little benzene and a little of this solution was added to a hot solution of lead tetraacetate (10·95 g.) in benzene (100 ml.). When the initially colloidal separation of lead diacetate had crystallized, the remaining acid was added and the mixture was boiled for a few minutes on the water bath. Liberation of CO_2 was demonstrated at this stage by trapping the evolved gas in baryta. The nearly colourless solution was washed with water and with N-HCl. A precipitate of $PbCl_2$ was removed and the benzene solution after further washing with water was extracted with 0·5 M-$NaHCO_3$. The alkaline extracts were extracted with ether; the ether extract was washed with water and with saturated aq. NaCl and added to the benzene solution, which had also been washed with NaCl. On removal of solvent *in vacuo* a yellow oil (4·05 g.), containing 2-bromo-4:8-dimethylnonan-1-al, remained. The alkaline extracts afforded 2·53 g. of acidic material.

To the crude bromo-aldehyde (4 g.) were added dry methanol (12 ml.) and ethyl orthoformate (3 ml.), followed by dry methanol saturated with HCl (0·25 ml.). Next day the solution was treated with sodium methoxide until neutral. Potassium hydroxide (0·7 g.) was then added and, after 1·5 hr., ether and water. The ether-soluble material (2·9 g.) was distilled to give 1·9 g., b.p. 83–85°/0·5 mm. (Found: C, 55·2; H, 9·1; Br, 22·9. $C_{13}H_{27}O_2Br$ requires C, 53·2; H, 9·1; Br, 27·1%.) If all the bromine in this product is attributable to 2-bromo-4:8-dimethylnonan-1-al dimethyl acetal the purity is 85%. This product (1·8 g.) was refluxed in methanol (9 ml.) containing KOH (0·9 g.) for 40 hr. On

distillation of the neutral ether-soluble product a main fraction (0·65 g.) was obtained, b.p. about 100°/15 mm. This showed no absorption at 223 mμ. in aq. ethanol, but a maximum at this wavelength appeared rapidly on addition of a trace of HCl. Acetic acid (1·8 ml.) was added to the crude acetal (0·65 g.) in ethanol (24 ml.) and water (24 ml.); after 5 min. a solution of semicarbazide hydrochloride and NaOAc,$3H_2O$ (400 mg. each) in a little water was added. Crystallization set in after a few minutes; the product was collected next day and recrystallized from 50 % aq. ethanol to give a first crop of 119 mg. Slow recrystallization from the same solvent produced large plates, m.p. 133–135° (Kofler), $[\alpha]_D^{20}$ – 22° (c = 1 in dioxan), of (–)-4:8-*dimethylnon-2-en-1-al semicarbazone*. (Found: C, 64·2; H, 9·9; N, 18·5. $C_{12}H_{23}ON_3$ requires C, 64·0; H, 10·2; N, 18·7 %.) The evidence for identity of this specimen with the semicarbazone from cholesterol has already been given (Cornforth *et al.* 1954). This semicarbazone absorbed maximally at 265 mμ. in ethanol. On being kept overnight with a solution of pyruvic acid in 85 % acetic acid, the aldehyde was regenerated as a pleasant-smelling oil showing intense absorption at 223 mμ.

At this stage of the work, a report from Zürich appeared (Riniker, Arigoni & Jeger, 1954) in which the preparation of the dihydro derivative of the aldehyde (IX) from cholesterol and from citronellal was described. Since the Swiss workers gave a procedure for ozonolysis of cholest-14-en-3β-yl benzoate and subsequent pyrolysis which seemed more convenient than our own, we adopted their method. By pyrolysis of the keto aldehyde (VIII; R = Bz) a distillate containing the aldehyde (IX) was obtained, and from the residue after saponification, chromatography and acetylation the tricyclic acetoxyketone (XVIII) was separated. Since this ketone has been obtained by unambiguous total synthesis (Cardwell, Cornforth, Duff, Holtermann & Robinson, 1951; Billeter & Miescher, 1950) both fragments of the pyrolysis of (VIII) are securely identified. A crystalline by-product which was present in the non-volatile residue before saponification appeared to be the lactone (XIX); this may have been formed from the keto aldehyde (VIII) by internal oxidation–reduction.

We now desired the unsaturated acid corresponding to (IX), in the expectation that its potassium salt (XX) would undergo αβ-fission on heating with potassium hydroxide, a reaction often put to good use in degradation (Hunter & Popjak, 1951; Cornforth *et al.* 1953*a*; Cornforth & Popjak, 1954). Experiments with synthetic aldehyde (IX) had shown this to resist oxidation by silver oxide, and since it was desirable to avoid more vigorous and less specific oxidizing agents we had recourse to a little-known method peculiarly suited to our purpose. The smooth cleavage of an aldoxime by hot aqueous alkali to give ammonia and the salt of a carboxylic acid:

$$R.CH{:}N.OH + KOH \rightarrow R.CO_2K + NH_3$$

seems first to have been applied by Zanetti (1891) to the succinaldoximes obtainable from pyrroles. The method appears to be rather general: we find, for example, that acetaldoxime is rapidly converted by boiling 30 % potassium hydroxide solution into ammonia and potassium acetate. When the aldehyde (IX) was treated with hydroxylamine and the crude oxime was heated with potassium hydroxide, ammonia was evolved at 100–150°. The temperature was then raised to 350° for a few minutes, when the expected fission of the αβ-unsaturated potassium salt of (XX) took place, the steam-volatile acidic product being shown by gas–liquid chromatography (James & Martin, 1952) to consist entirely of acetic acid and a branched-chain C_9 acid. The process was now repeated with radioactive material; the two acids (XXI) and (XXII) were separated by partition chromatography and shown to occur in nearly equimolecular amount and in about 70 % yield based on the alkali consumed during oximation. These two acids were reserved for isolation of carbon atoms 15, 16 and 17 of cholesterol (see below). Their structure and origin follow unambiguously from the mode of formation, the composition of the silver salt of (XXI), and the gas–liquid chromatographic analysis.

The radioactive acetoxyketones (XVIII; R = Ac) were diluted with an equal amount of non-radioactive product (prepared from the 5:6-dehydro analogue derived from oxidation of cholesteryl acetate dibromide and generously supplied by Dr K. Miescher of Ciba Laboratories, Basle, Switzerland) and degraded further by a procedure based on that of Billeter & Miescher (1950). Bromination in benzene solution, followed by dehydrobromination with pyridine, gave a product consisting largely of the unsaturated ketone (XXIII). This always contained a small proportion of the saturated ketone (XVIII; R = Ac) even when the pure bromide (XXIV) was isolated as an intermediate, some reversal of the bromination presumably being effected by pyridine hydrobromide; complete purification of the ketone (XXIII) would have been expensive but was fortunately unnecessary. Ozonization in ethyl chloride at – 30°, followed by steam-distillation of the product, gave a distillate containing a 50–60 % yield of acetic acid and a trace of formic acid; the two acids were identified by gas–liquid chromatography. The acetic acid was purified by partition chromatography and was reserved for identification of carbon atoms 18 and 13 of cholesterol. Its origin from these two carbons cannot be doubted: the acetoxyl group in (XXIII) should not be appreciably hydrolysed under these conditions, nor could acetic acid thus formed have the radioactivity later detected.

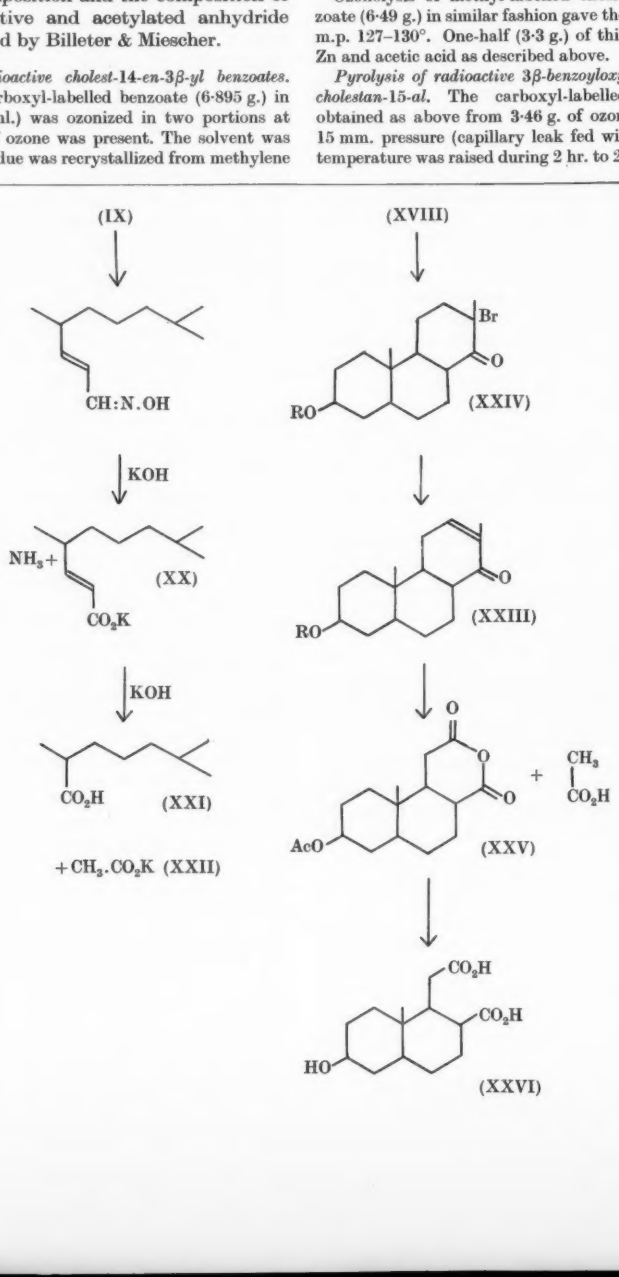
The product non-volatile in steam was further oxidized by hydrogen peroxide; treatment of the

acidic fraction with acetic anhydride then gave the anhydride (XXV) described by Billeter & Miescher (1950), who oxidized the unsaturated ketone (XXIII) with chromic acid in acetic acid. This anhydride on alkaline hydrolysis afforded the hydroxydicarboxylic acid (XXVI), which served as the source of carbons 12 and 14 of cholesterol (see below). Its structure follows from its mode of formation, its composition and the composition of the acetyl derivative and acetylated anhydride previously obtained by Billeter & Miescher.

Ozonolysis of radioactive cholest-14-en-3β-yl benzoates. A solution of the carboxyl-labelled benzoate (6·895 g.) in ethyl chloride (100 ml.) was ozonized in two portions at −70° until excess of ozone was present. The solvent was removed and the residue was recrystallized from methylene chloride by addition of methanol to give the ozonide (6·92 g.), m.p. 128–130°. One-half (3·46 g.) of this product was stirred for 2 hr. on a steam bath with acetic acid (170 ml.) and Zn powder (11·5 g.). The cooled filtered solution was evaporated at low pressure; the residue was dissolved in ether and water. The ethereal layer, after washing with dilute NaOH and aq. NaCl, was dried and evaporated. The crude keto aldehyde (VIII) was used directly for the next stage.

Ozonolysis of methyl-labelled cholest-14-en-3β-yl benzoate (6·49 g.) in similar fashion gave the ozonide (6·635 g.), m.p. 127–130°. One-half (3·3 g.) of this was reduced with Zn and acetic acid as described above.

Pyrolysis of radioactive 3β-benzoyloxy-14-oxo-14:15-seco-cholestan-15-al. The carboxyl-labelled keto aldehyde, obtained as above from 3·46 g. of ozonide, was heated at 15 mm. pressure (capillary leak fed with N_2) to 200°; the temperature was raised during 2 hr. to 250° and maintained



there for 3 hr. The volatile product, trapped in a receiver at −30°, was taken up in a little ether and washed with a few drops of 0·5 M-$NaHCO_3$ to remove benzoic acid. The ether was removed at low pressure and the residue was distilled, b.p. about 110°/15 mm., to give the aldehyde (IX) as a colourless pleasant-smelling oil (251 mg.).

Similar treatment of the methyl-labelled keto aldehyde (from 3·3 g. of ozonide) gave 181 mg. of redistilled aldehyde (IX).

Degradation of radioactive 4:8-*dimethylnon*-2-*en*-1-*al to give acetic acid and* 2:6-*dimethylheptanoic acid.* To the carboxyl-labelled aldehyde (251 mg.) was added hydroxylamine hydrochloride (100 mg.) with a few drops of water and of propan-2-ol and a trace of methyl orange. N-Sodium hydroxide solution was then added slowly as oximation proceeded until the red colour of the indicator failed to return: 1·35 ml. was required (90% of the theoretical). Propan-2-ol was added from time to time to dissolve most of the oil which separated (ethanol instead of propan-2-ol was found in pilot experiments to produce unwanted acetic acid by oxidation during the subsequent heating). The ether-soluble product, after washing with water, was treated with KOH pellets (2·5 g.), a solid yellow potassium salt being formed. The temperature was slowly raised to 350° and kept there for 10 min. Ammonia was evolved from 110–200°. The cooled melt was dissolved in water, acidified with H_2SO_4 and distilled in steam. The distillate was neutralized with NaOH (phenol red) and evaporated at low pressure. The sodium salts were decomposed by addition of 15 N-H_2SO_4 (0·2 ml.); this mixture on a pad of asbestos was placed at the top of a chromatographic column in which 0·5 N-H_2SO_4 supported by Hyflo Super Cel was the stationary phase (cf. Cornforth, Hunter & Popjak, 1953*b*). The column was developed first with $CHCl_3$, successive 5 ml. portions of effluent being titrated with 0·19 N ethanolic LiOH (bromothymol blue). The 2:6-dimethylheptanoic acid ran fast through the column and required 5·17 ml. (0·982 m-mole) for neutralization. Thereafter, $CHCl_3$ containing 10% (v/v) of BuOH was used to elute the acetic acid, which required 6·24 ml. (1·185 m-moles). The lithium salts were recovered by evaporation; that of 2:6-dimethylheptanoic acid was redissolved in water and the silver salt was precipitated by gradual addition of $AgNO_3$, the first portion of precipitate being rejected. The silver salt was dried *in vacuo* over P_2O_5. (Found: for a non-radioactive specimen, C, 40·55; H, 6·9; residue 42·1. $C_9H_{17}O_2Ag$ requires C, 40·8; H, 6·4; Ag, 40·8%.)

Methyl-labelled dimethylnonenal (181 mg.) was put through the same process. During oximation, 0·93 ml. of N-NaOH was consumed (86%). The steam-volatile acids obtained after fusion of the oxime were resolved into dimethylheptanoic acid (0·606 m-mole) and acetic acid (0·725 m-mole).

Radioactive 2:13-*dimethyl*-trans-anti-trans-*perhydrophenanthren*-1-*on*-7β-*yl acetates.* The non-volatile product of pyrolysis from carboxyl-labelled keto aldehyde (VIII), dissolved in a little benzene, was refluxed with 0·5 N methanolic KOH (23 ml.) for 1 hr. The neutral product, recovered in the normal manner, was dissolved in benzene and put on a column of alumina. The column was washed with benzene and then with benzene–ether (9:1). After elution of some gummy material, partly crystalline fractions were obtained totalling 1·35 g. These were combined and acetylated by leaving for 24 hr. with acetic anhydride (5 ml.) and pyridine (2·5 ml.). The neutral product was separated in the usual way and recrystallized from light petroleum (b.p. 40–60°) and then from methanol containing a few drops of water. The acetoxyketone (XVIII, R = Ac) (339·4 mg.) then had m.p. 144–145°.

In a similar fashion the pyrolysate from methyl-labelled keto aldehyde afforded acetoxyketone (XVIII; R = Ac) (297·0 mg.) m.p. 144–145°.

Each of these specimens was mixed with an equal weight of non-radioactive acetoxyketone, m.p. 144–145°, prepared according to Billeter & Miescher (1950).

3β-*Benzoyloxy*-14-*hydroxy*-14:15-seco*cholestan*-15-*oic acid lactone.* After pyrolysis of a portion (2·92 g.) of non-radioactive keto aldehyde (VIII) the non-volatile product in benzene was chromatographed directly on alumina (previously neutralized by washing with methyl formate). Benzene eluted the benzoate of the tricyclic ketone; thereafter, benzene–ether (3:1) eluted a product which after two recrystallizations from ethanol formed colourless needles (0·11 g.), m.p. 183·5°. (Found: C, 78·2; H, 9·7. $C_{34}H_{50}O_4$ requires C, 78·2; H, 9·6%.) The infrared spectrum (in compressed KBr) showed carbonyl absorption at 1713 and 1745 cm.$^{-1}$. This confirmed that the *lactone* (XIX) had been isolated.

Radioactive 2:13-*dimethyl*-trans-anti-trans-1:4:5:6:7:8:9:10:11:12:13:14-*dodecahydrophenanthr*-1-*on*-7β-*yl acetates.* Carboxyl-labelled acetoxyketone (XVIII; R = Ac; 0·6788 g.) was dissolved in thiophen-free benzene (25 ml.), cooled in ice and treated gradually with a solution of bromine in benzene, each portion being allowed to react before adding the next (5·65 ml. of 0·845 N). The solution was washed with 0·5 M-$NaHCO_3$ and the benzene was evaporated at low pressure. The crystalline residue was boiled with pyridine (4·5 ml.) for 10 min. and the solution was poured into 2 N-HCl (30 ml.). The neutral product was isolated and crystallized at −5° from hexane to give the unsaturated ketone (XXIII) (0·54 g.), m.p. 101–103·5°.

The methyl-labelled analogue (0·525 g.; m.p. 102–105°) was prepared similarly from the saturated ketone (0·5940 g.).

Ozonolysis of radioactive unsaturated ketone (XXIII). Carboxyl-labelled unsaturated ketone (0·500 g.) was dissolved in ethyl chloride (15 ml., previously treated with ozone and washed with 0·5 M-$NaHCO_3$), cooled to −30° and ozonized for 3 hr. Ozone was still present in solution after keeping at −70° overnight. The ozone was displaced by a current of air and the solvent was evaporated. The residue after warming with a little water was distilled in steam. The distillate after neutralization with NaOH was evaporated; vapour-phase chromatography of the acidified residue indicated the presence of acetic acid and a trace of formic acid. Partition chromatography by the technique already described (5% butanol in $CHCl_3$ was used) separated the acetic acid (1·056 m-moles), which was reserved as the lithium salt.

Ozonolysis of the methyl-labelled analogue (0·4118 g.) in the same manner yielded acetic acid (0·725 m-mole).

Radioactive 1-*carboxymethyldecahydro*-6-*hydroxy*-9-*methyl*-2-*naphthoic acids.* The fraction non-volatile in steam, from ozonization of carboxyl-labelled unsaturated ketone (500 mg.), was extracted with ether and $CHCl_3$; it weighed 450 mg. This was dissolved in acetic acid (3 ml.) and the solution was added slowly to a mixture of 30% H_2O_2 (0·3 ml.), water (0·6 ml.) and conc. H_2SO_4 (0·015 ml.); the whole was then refluxed for 1 hr., diluted with water

(16 ml.) and extracted thrice with ether. The ether layer was washed with water and the solvents were evaporated, finally *in vacuo* over KOH. The residue was dissolved in ether and extracted with 3×5 ml. of 2N-NaOH. On evaporation of the ether, a residue (19·7 mg.) remained which consisted chiefly of the saturated ketone (XVIII; R = Ac). The alkaline extract was acidified to Congo red with 2N-HCl and extracted continuously with ether. The extracted product (208 mg.) was dissolved in dry pyridine (1·7 ml.) and acetic anhydride (0·85 ml.). Next day the mixture was evaporated at low pressure, ether and water were added, and the ether, after washing with 0·5M-$NaHCO_3$ and water, was evaporated. The residue (185 mg.) crystallized in contact with CCl_4. It was left for some months at this stage, during which time a little hydrolysis occurred; this was corrected by warming with acetic anhydride (1 ml.) at 100° for 1 hr. After concentration at low pressure the product was recrystallized from CCl_4 to give 6-acetoxy-1-carboxymethyldecahydro-9-methyl-2-naphthoic acid anhydride (83 mg.) in prisms, m.p. 193°. This material was boiled for 2 hr. with 0·5N-NaOH (2·00 ml.); N-HCl (1 ml.) was then added and the solution was quickly concentrated at low pressure. The *acid* (XXVI) crystallized in small colourless prisms (70 mg.). [Found for a similarly prepared non-radioactive specimen: m.p. 234–236° (decomp.); $[\alpha]_D^{23} + 4°$ [c = 0·5 in $CHCl_3$–ethanol]. Found: C, 62·1; H, 8·4. $C_{14}H_{22}O_5$ requires C, 62·2; H, 8·2%.]

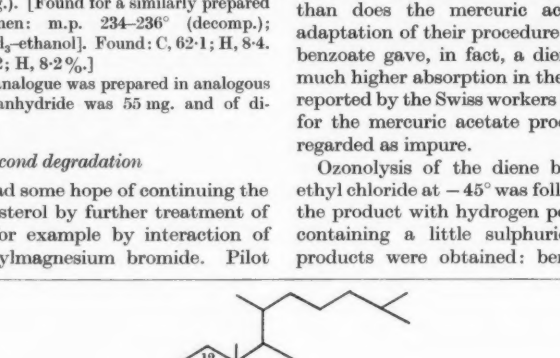
The methyl-labelled analogue was prepared in analogous fashion; the yield of anhydride was 55 mg. and of dicarboxylic acid 46 mg.

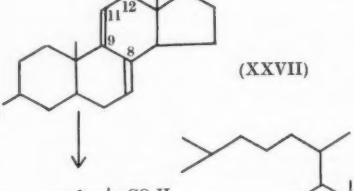
The second degradation

At this stage we had some hope of continuing the degradation of cholesterol by further treatment of the acid (XXVI), for example by interaction of an ester with phenylmagnesium bromide. Pilot experiments with non-radioactive material showed, however, that interaction with both ester groups was difficult to attain, and finally we turned to a different plan. The execution of this required more radioactive 7-dehydrocholesterol; in order to save the labour of repeating this preparation we recrystallized non-radioactive 7-dehydrocholesterol in the mother liquors from which radioactive 7-dehydrocholesterol had separated. This expedient afforded material of low but sufficient radioactivity. Benzoylation, followed by hydrogenation in benzene over Raney nickel, then gave cholest-7-en-3β-yl benzoate (III; R = Bz).

The preparation of cholesta-7:9(11)-dien-3β-yl benzoate (XXVII) by oxidation of a 7-ene with mercuric acetate has twice been reported (Fieser, Herz & Huang, 1951; Heusser, Heusler, Eichenberger, Honegger & Jeger, 1952). Anderson, Stevenson & Spring (1952) have shown in the ergostane series that dehydrogenation by bromine (followed by sodium iodide) gives purer products than does the mercuric acetate method. Our adaptation of their procedure to cholest-7-en-3β-yl benzoate gave, in fact, a diene (XXVII) showing much higher absorption in the ultraviolet than was reported by the Swiss workers (and confirmed by us) for the mercuric acetate product, which must be regarded as impure.

Ozonolysis of the diene benzoate (XXVII) in ethyl chloride at −45° was followed by treatment of the product with hydrogen peroxide in acetic acid containing a little sulphuric acid. Three main products were obtained: benzoic acid, a water-





soluble acid slowly extractable by ether, and a light-petroleum-soluble acid precipitable as an amorphous sodium salt.

The water-soluble acid appeared to be a mixture of the lactone–acid (XXVIII) and the corresponding dicarboxylic acid, for when it was melted at 180° water was expelled and the high-melting lactone–acid crystallized and could be purified by sublimation. The structure (XXVIII) for the lactone–acid is established by its composition and properties; in particular, an isomeride in which C-9 (of cholesterol) is lactonized and C-7 free is stereochemically impossible without an (unlikely) inversion at C-3 or C-10.

The acid from the insoluble sodium salt was converted into an anhydride, which was distilled; the regenerated acid, itself an oil, was purified by crystallization of its ammonium salt, for which analysis indicated the formula (XXIX). This attribution is supported by the following arguments: (1) a dicarboxylic acid of this structure is the expected product of ozonolysis under the mild conditions employed, and is complementary to the lactone–acid already isolated; (2) the only other acid of at all similar composition which can be postulated to arise from this ozonolysis is the nor-dicarboxylic acid (XXX), and the analytical data do not agree with this interpretation.

A rather similar but less direct fission of the ergosterol ring system has been described (Heusser, Beriger, Anliker, Jeger & Ruzicka, 1953), nine stages from the 7:9(11)-diene being required to disrupt the molecule.

Radioactive cholesta-5:7-dien-3β-yl benzoates. The mother liquor from which carboxyl-labelled cholesta-5:7-dien-3β-ol had separated (see above) was evaporated. To the residue (1·37 g.) non-radioactive cholesta-5:7-dien-3β-ol (3·10 g.) was added in ether and the mixture was evaporated. The residue was dissolved in pyridine (21 ml.) and benzoyl chloride (3 ml.) was added. After 60 hr., acetone (6·7 ml.) and water (3·4 ml.) were added; ½ hr. later the mixture was poured into water and extracted with $CHCl_3$. The neutral product, recovered in the usual manner, was boiled with acetone, cooled and collected, to give 3·189 g., m.p. 139–140°, ϵ_{282} 12800.

The residue (1·54 g.) from the mother liquor of methyl-labelled cholesta-5:7-dien-3β-ol was diluted with non-radioactive dienol (3·11 g.) and the product was benzoylated as above to give 3·225 g., m.p. 139–140°, ϵ_{282} 12950.

Radioactive cholest-7-en-3β-yl benzoates. The carboxyl-labelled diene benzoate (3·18 g.) in benzene (200 ml.) was hydrogenated over Raney nickel for 16 hr. in the manner described above for the corresponding alcohol. The product was recrystallized from acetone to give cholest-7-en-3β-yl benzoate (2·516 g.), m.p. 155° (clear at 174°).

The methyl-labelled analogue (2·60 g.) was obtained similarly; m.p. 156° (clear at 176°).

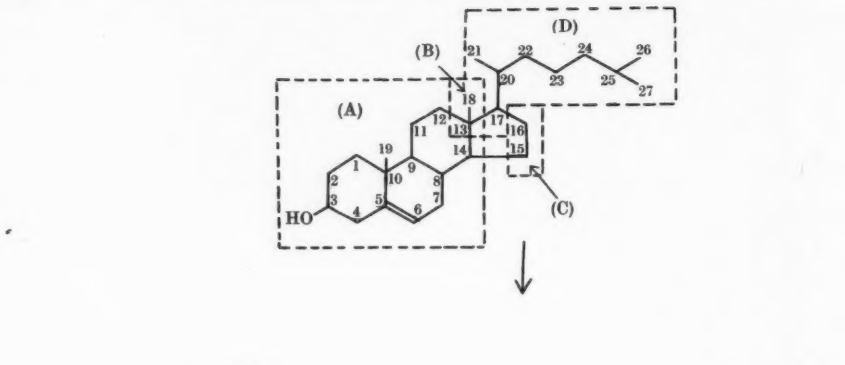
Radioactive cholesta-7:9(11)-dien-3β-yl benzoates. To carboxyl-labelled cholest-7-en-3β-yl benzoate (2·50 g.) in dry ether (250 ml.) at 0° was rapidly added dry bromine (0·825 ml.) in acetic acid (8·25 ml.). The mixture was cooled quickly to −60°, then warmed gradually to 0° during 2 hr. and concentrated somewhat at low pressure. The solid bromo compound (2·2 g.) was collected, dissolved in warm benzene (150 ml.) and treated with NaI (8 g.) in ethanol (150 ml.). Iodine was liberated immediately. After 20 hr. water (150 ml.) was added. The aqueous layer was washed with benzene (100 ml.) and the combined benzene solutions were shaken with 0·25 N-NaOH (2 × 60 ml.), washed with water and evaporated at low pressure. The residue, after two crystallizations from chloroform–methanol, gave cholesta-7:9(11)-dien-3β-yl benzoate (1·458 g.) m.p. 135–136°, showing maximum absorption at 234 mμ. (ε 31500).

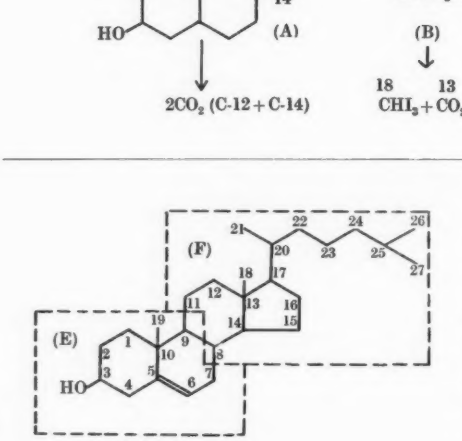
By the same procedures, methyl-labelled cholest-7-en-3β-yl benzoate (2·6 g.) gave cholesta-7:9-dien-3β-yl benzoate (1·406 g.), m.p. 136–136·5°, ϵ_{234} 30000. A non-radioactive specimen prepared similarly had $[\alpha]_D^{22.5}$ +48°. (Found: C, 83·8; H, 9·9. Calc. for $C_{34}H_{48}O_2$: C, 83·6; H, 9·9%.) Heusser *et al.* (1952) gave m.p. 132–133·5°, $[\alpha]_D^{19}$ +52°, and maximum absorption at 228 mμ., ε 19950, for material prepared by means of mercuric acetate. Fieser *et al.* (1951) gave m.p. 134°, $[\alpha]_D$ +32° (dioxan).

Ozonolysis of radioactive cholesta-7:9(11)-dien-3β-yl benzoates. Carboxyl-labelled diene benzoate (1·4 g.) was dissolved in ethyl chloride (77 ml.) and ozonized at −45° until the solution remained blue. Ozone was displaced by a stream of air and the solvent was removed. The residue was dissolved in acetic acid (11·2 ml.) and added gradually to 30% H_2O_2 (1·12 ml.) in water (2·24 ml.) and concentrated H_2SO_4 (0·07 ml.). The mixture was refluxed for 2·5 hr. and cooled. After addition of $NaOAc,3H_2O$ (170 mg.) the solvents were removed at low pressure, finally at 0·5 mm. To the residue were added ether and 2 N-NaOH (100 ml.). A sodium salt separated in the ether and was dissolved by washing twice with water. The combined solutions were acidified to Congo red with HCl and extracted twice with ether (total about 50 ml.). The aqueous layer (*A*) and the ethereal layer (*B*) were separated.

Layer *A* was extracted continuously with ether for 48 hr. The extracted product was dissolved in acetone, concentrated to crystallizing point and treated with about 4 vol. of benzene. The product, m.p. 150–160°, was collected, washed with acetone–benzene (1:3) and heated to 180–190°/100 mm. Effervescence ceased after about 10 min. and the melt solidified. A cold-finger condenser was inserted; the pressure was lowered to 15 mm. and the temperature raised to 250°. The product sublimed rapidly; it was dissolved in the minimum (30 ml.) of boiling xylene. After cooling, the large, blade-like crystals were collected and washed with benzene to give 183 mg., m.p. 248–250°, of *6-methyl-2-oxabicyclo-[3:3:1]-nonan-3-one-6-carboxylic acid* (XXVIII). A non-radioactive specimen had m.p. 248–250°, $[\alpha]_D^{26}$ −12·5°. (Found: C, 60·9; H, 6·9%; equiv. 198. $C_{10}H_{14}O_4$ requires C, 60·6; H, 7·1%; equiv. for 1 CO_2H, 198). The infrared spectrum in compressed KCl showed maxima at 1720 cm.$^{-1}$ (lactone CO) and 1680 cm.$^{-1}$ (carboxyl CO).

Layer *B* was evaporated and benzoic acid was sublimed from the residue at 100°/15 mm. To the remainder, 2·5 N-NaOH (10 ml.) was added and the mixture warmed for 1 hr. on a steam bath. The sodium salt (which had separated almost at once) was collected from the hot solution, washed with warm 2·5 N-NaOH and dissolved in water. The free acid was liberated and extracted with light petroleum (b.p. 40–60°); evaporation of the solvent left an oil (263 mg.) which





was boiled for 45 min. with acetic anhydride (4 ml.). The product was recovered by evaporation and distilled at 150–200° (heating block) and 0·04 mm. The distillate (188 mg.) was hydrolysed by warming for 4 hr. on a steam bath with 0·1 N-NaOH (13·2) and the acid (203 mg.) was recovered by means of light petroleum. It was dissolved in a minimum of dilute aq. NH_3; concentrated aq. NH_3 (sp.gr. 0·880) was then added, when the *diammonium salt of* 1-(1:5-*dimethylhexyl*)-2-*methyl*cyclo*pentane*-2-*carboxymethyl*-3-*carboxylate* (XXIX) crystallized in fine colourless needles (144 mg.), which were collected by centrifuging at 0° and dried over KOH in an atmosphere containing NH_3. A non-radioactive specimen had $[\alpha]_D^{23\cdot5} + 50°$ (c = 1 in water). (Found: C, 61·8; H, 10·8; N, 8·0. $C_{17}H_{36}O_4N_2$ requires C, 61·4; H, 10·8; N, 8·4%.) An ammonium salt of the nor-dicarboxylic acid (XXX), $C_{16}H_{34}O_4N_2$, requires C, 60·4; H, 10·7; N, 8·8%. The salt melted and decomposed at about 100°, and was unstable *in vacuo*, losing NH_3 and reverting to a gum.

Methyl-labelled cholesta-7:9(11)-dien-3β-yl benzoate (1·37 g.) was ozonized and worked up in essentially the same manner, but less profitably, 120 mg. of lactone (XXVII), m.p. 248–250°, and 88 mg. of ammonium salt (XXIX) being obtained.

Further degradation to one-carbon fragments

Having now recorded the cleavage of cholesterol into six fragments of convenient structure, we shall describe the separation from these of the carbon atoms whose biosynthetic origin was to be determined. The progress from cholesterol to the six

fragments lettered (A) to (F), and thence to one-carbon compounds representing various positions in the molecule of cholesterol, is shown diagrammatically on p. 104.

The two specimens of lithium acetate (B and C) were converted into barium carbonate (representing the carboxyl groups) and iodoform (representing the methyl groups) by a well-established procedure (cf. Popjak, 1955). The silver salt (D) of the C_9 acid (XXIII) on treatment with bromine readily gave up carbon dioxide, which was collected as barium carbonate. Thus carbon atoms 13, 15, 16, 17 and 18 of cholesterol had been isolated.

The lactone–acid (E), on warming in concentrated sulphuric acid at 95°, liberated carbon monoxide, which was oxidized with iodine pentoxide to carbon dioxide and finally converted into barium carbonate. The yield was 60–70 %. Loss of carbon monoxide under these conditions is a characteristic of tertiary carboxylic acids, as was shown originally by Bistrzycki (cf. Bistrzycki & Mauron, 1907, and earlier papers there cited); thus it is certain that this carbon monoxide derived ultimately from C-9 of cholesterol and not from C-7, the other (potential) carboxyl group being primary.

More difficulty was caused by the dicarboxylic acids (A and F). Although the carboxyl groups in these acids are non-equivalent and procedures could no doubt be devised to eliminate them separately, lack of material made us decide for complete decarboxylation. The carbon dioxide from each acid would then represent two different carbon atoms in the molecule of cholesterol. Hence the biosynthetic origin of the individual carbon atoms could not be deduced from measurements of radioactivity on this carbon dioxide unless two conditions were satisfied. First, the yield of carbon dioxide must be high enough to make certain that both carboxyl groups contributed to it. Secondly, if the carbon dioxide was inactive (or nearly so) when an acid deriving from one type of cholesterol (e.g., methyl-labelled) was decarboxylated, then decarboxylation of the acid from the other type of cholesterol must give radioactive carbon dioxide having approximately the degree of radioactivity expected for labelled positions. Had the carbon dioxide from both types of cholesterol been radioactive, or had an unexpected level of radioactivity been found, it would then have been necessary to take the carboxyl groups separately.

The Schmidt reaction was tried first, but it was found impossible, despite many variations in technique, to raise the yield of carbon dioxide from either dicarboxylic acid above 50 % of the theoretical for two carboxyl groups. Bromine on the silver salt of dicarboxylic acid (F) gave only a 30 % yield of carbon dioxide. The method finally adopted was that of anodic decarboxylation. Electrolysis of carboxylic acid salts has been known for more than a century to cause decarboxylation; an excellent review is given by Weedon (1952). The process can logically be formulated as the discharge of a carboxylate ion and decomposition of the carboxylate radical to give carbon dioxide and an alkyl radical which then dimerizes, disproportionates or reacts with another molecule.

$$R.CO_2^- - e \rightarrow R.CO_2^{\cdot} \rightarrow R^{\cdot} + CO_2.$$

It is often difficult to obtain a high yield of an individual product arising from the radical $R^{\cdot}$, but the only known side reaction which diminishes the yield of carbon dioxide is that which leads to an ester,

$$2R.CO_2^- - 2e \rightarrow R.CO_2R + CO_2,$$

and this is usually of little import.

For the present purpose, only the carbon dioxide was required. The acid (A) was electrolysed in aqueous pyridine, the acid (F) in aqueous pyridine containing a little triethylamine to improve conductivity. The yield of carbon dioxide isolated as barium carbonate was 70 % for A, and 75–80 % for F, of the theoretical value for two carboxyl groups.

Degradation of lithium acetate specimens [*(B) = carbons* 18 + 13] *and* [*(C) = carbons* 16 + 15]. These were carried out by pyrolysis *in vacuo* (10^{-4} mm. Hg) to give finally $BaCO_3$ from the CO_2H and CHI_3 from the methyl carbons. From lithium acetate (B) of carboxyl-labelled cholesterol 75 mg. of CHI_3 (C-18) and 41 mg. of $BaCO_3$ (C-13), and from methyl-labelled cholesterol 63 mg. of CHI_3 and 36·6 mg. of $BaCO_3$, were obtained. Lithium acetate (C) from carboxyl-labelled cholesterol gave 106 mg. of CHI_3 (C-16) and 63·6 mg. of $BaCO_3$ (C-15), and from methyl-labelled cholesterol 37 mg. of CHI_3 and 38 mg. of $BaCO_3$ (the centrifuge tube in which this sample was being washed broke and the CO_2 had to be regenerated *in vacuo* and trapped in fresh baryta).

Decarboxylation of silver salts (D). To a fine suspension of the dry carboxyl-labelled silver salt (150 mg.) in dry CCl_4 (2 ml.) was added bromine (0·09 g.) in CCl_4 (0·3 ml.). Carbon dioxide was evolved and, after passing a short air condenser, was swept by CO_2-free N_2 into half-saturated $Ba(OH)_2$. Finally the suspension was boiled to complete the reaction and to drive over the remaining CO_2. The yield of $BaCO_3$ was 61 mg. Methyl-labelled silver salt (120 mg.) gave 44 mg. of $BaCO_3$ by the same procedure.

Decarboxylation of lactone–acids (E). Carboxyl-labelled lactone–acid (72 mg.) was dissolved in concentrated H_2SO_4 (2 ml.) and heated to 95°. The liberated gas was swept by CO_2-free N_2 through soda-lime (to absorb SO_2 and CO_2), then through a tube of I_2O_5 maintained at 120°, and finally through half-saturated $Ba(OH)_2$. Iodine was formed from the I_2O_5 and $BaCO_3$ precipitated in the $Ba(OH)_2$. After 75 min. heating was discontinued; after a further 45 min. the $BaCO_3$ (44 mg.) was collected. Methyl-labelled lactone–acid (66·5 mg.) by the same procedure gave $BaCO_3$ (42·7 mg.).

Anodic decarboxylation of acids (A). The electrolytic cell was a small tube fitted by means of a B14 standard ground-glass joint to a cap carrying gas inlet and outlet tubes and

two platinum wires, sealed through the glass, which projected to the bottom of the cell and were maintained 3 mm. apart by a glass support fused around them. The cell was charged with carboxyl-labelled acid (A) (30·8 mg.) in pyridine (0·1 ml.) and water (0·2 ml.) and a potential of 12 v was applied to the electrodes. The cell was cooled externally by water and the gas evolved was swept by CO_2-free N_2 into half-saturated $Ba(OH)_2$. The initial current was 34 mA; this dropped steadily as carboxylate ions were eliminated, and after 62 min. was 6 mA; it was computed graphically that 52 coulombs had passed. The circuit was broken and the cell was heated in boiling water for ½ hr. The $BaCO_3$ after collection weighed 31·5 mg.

Methyl-labelled acid A (33 mg.) gave $BaCO_3$ (33 mg.) by the same procedure.

Anodic decarboxylation of acids (*F*). The ammonium salt (45·3 mg.) of carboxyl-labelled acid (F) was decomposed by ether and 0·1 N-HCl. The acid was transferred quantitatively to the electrolytic cell and dissolved in pyridine–water (3:1; 0·3 ml.). Triethylamine (0·025 ml.) was added and a potential of 24 v was applied. The initial current was 48 mA; this diminished during ½ hr. to a steady value of 18 mA. After 40 min. (61 coulombs) the current was stopped and the cell was heated in boiling water for ½ hr.; after cooling, only 7 mA passed on applying 24 v to the electrodes, showing that the triethylamine, unlike pyridine, had retained some CO_2 at room temperature. The $BaCO_3$ after collection weighed 40·1 mg. Methyl-labelled ammonium salt (43·4 mg.) gave $BaCO_3$ (42·3 mg.) in the same way.

Measurements of radioactivity. The samples of $BaCO_3$ and CHI_3 from acetic acids (B) and (C) and of $BaCO_3$ from the silver salt (D) were counted on plastic planchets (Popjak, 1950) with a thin mica-window counter. Carboxyl-labelled and methyl-labelled cholest-14-en-3β-yl benzoates were counted in the same way. Appropriate correction for the greater back-scatter of β-rays from the $BaCO_3$ and CHI_3 samples as compared with the cholestenyl benzoate samples were made. The counts for these samples are given in Table 1 as counts/min. at infinite thickness carbon, these being proportional to the specific activity of the carbon.

The $BaCO_3$ from the lactone–acids E and the dicarboxylic acids (A) and (F) was decomposed in Rittenberg tubes and the CO_2 was counted in a proportional gas counter (Bradley, Holloway & McFarlane, 1954). Carbon dioxide from combustion of acids (A), (E) and (F) was counted in the same apparatus. The counts obtained (Table 2) were calculated, on the basis of the known efficiency of the gas counters used, in terms of number of disintegrations/min./mg. of carbon.

RESULTS AND DISCUSSION

Degradation of ring D and identification of carbons 13, 15, 16, 17 and 18 were completed in 1954, and the results have been briefly reported (Cornforth, 1954; Cornforth, Popjak & Gore, 1956). Table 1 shows that of these five carbon atoms, only one, C-16, originates from the carboxyl carbon of acetate and all the others from the methyl carbon. When [*carboxy*-^{14}C]acetate was the precursor of cholesterol C-13, C-15, C-17 and C-18 contained no detectable radioactivity, as previously reported (Cornforth *et al.* 1953*b*) for C-1, C-3, C-5 and C-10. On the other hand, when [*Me*-^{14}C]acetate was the precursor, two types of labelling were found: one with high radioactivity (C-13, C-15, C-17 and C-18) and another (C-16) with low but significant activity. This duality was also found in the earlier work with ring A and was attributed to the conversion of some [*Me*-^{14}C]acetate into [*carboxy*-^{14}C]acetate by way of the tricarboxylic acid cycle; in the present experiments, however, this effect was less marked than before. As mentioned earlier, the same biosynthesis from [*Me*-^{14}C]acetate which provided us with methyl-labelled cholesterol was also used to prepare methyl-labelled squalene, the degradation of which has been reported (Cornforth & Popjak, 1954). The same two types of labelling were found in this squalene, and the ratio of the radioactivities was close to that now found in cholesterol.

In the second set of degradations, aiming at C-9, C-11, C-8, C-12 and C-14, it has not been possible to relate all the results to a single parent compound, as some of the fragments from which these carbon atoms were obtained have of necessity been diluted to different and unpredictable degrees in the course

Table 1. *Specific activity* (*counts/min./mg. of* C) *of carbons* 15, 16, 17, 18 *and* 13 *of cholesterol biosynthesized from* [carboxy-^{14}C]*acetate and from* [Me-^{14}C]*acetate*

Precursor of cholesterol ...	Counts/min./mg. of C: $CH_3.{}^{14}CO_2H$*	Counts/min./mg. of C: $^{14}CH_3.CO_2H$*
Cholest-14(15)-enol	568	733
C-15	Inactive	1080†
C-16	1336	98
C-17	Inactive	1279
C-18	Inactive	1386
C-13	Inactive	1271

* The two samples of cholesterol were biosynthesized by two different batches of liver slices, and were diluted by inactive material to a different degree. The specific activity of the labelled carbon atoms in the two samples, calculated on the assumption that 12 carbon atoms of cholesterol originate from the carboxyl carbon and 15 from the methyl carbon of acetate, were 1275 counts/min./mg. of C (precursor: $CH_3.{}^{14}CO_2H$) and 1238 counts/min./mg. of C (precursor: $^{14}CH_3.CO_2H$).

† This sample of $BaCO_3$ was recovered from the accident mentoned above (p. 105).

Table 2. *Specific activity* (*disintegrations/min./mg. of* C) *of* C-9, C-11 + C-8, *and of* C-12 + C-14 *and of the total carbon of the fragments from which they originated*

Precursor of cholesterol ...	Disintegrations/min./mg. of C: $CH_3.{}^{14}CO_2H$	Disintegrations/min./mg. of C: $^{14}CH_3.CO_2H$
Lactone acid (fragment E)	5·1	17·8
C-9 (from fragment E)	Inactive	26·8
Dicarboxylic acid (fragment F)	5·7	16·0
C-8 + C-11 (from fragment F)	11·0	1·1
Dicarboxylic acid (fragment A)	330·0	277·0
C-12 + C-14 (from fragment A)	508·0	19·9

of chemical manipulation. Instead, we present in Table 2 specific activities of the total carbon of the various fragments and of the individual carbon atoms derived from these.

The results collected in Table 2 show that C-9 is a methyl carbon and that the other carbons, C-8, C-11, C-12 and C-14, are carboxyl carbons. If we denote a methyl carbon by m and a carboxyl carbon by c, the now complete pattern in cholesterol is as shown (below).

Additional evidence for the origin of the four carbons, C-8, C-11, C-12 and C-14 was desirable, for the method of anodic decarboxylation by which they were separated is new in this context. Although the disappearance of carboxyl groups during electrolysis (as shown by the fall in conductivity) paralleled the evolution of CO_2, and although one can certainly conclude that all or nearly all this CO_2 originated from carboxyl carbons and none from methyl carbons, it was still possible to doubt that all the CO_2 came from the carboxyl groups of the parent acids. Further evidence not dependent upon this assumption was desirable, and was provided by examining the total radioactivity of the fragments (A), (E) and (F).

Fragment (A) is a product of the first degradation, the results of which are given in Table 1. We calculated the average specific activity of the three methyl carbons C-13, C-17 and C-18, and compared this with the specific activity of the carboxyl carbon C-16, obtaining the proportions given in Table 3.

We made the assumption, justified by the present and all previous work on degradation of cholesterol biosynthesized from acetate, that these proportions are substantially true of the other methyl and carboxyl carbons in these two specimens of cholesterol, and hence in the two specimens of fragment (A) which derive from them.

Now if (A) contains eight carboxyl and six methyl carbons, as indicated by the results in Table 2 taken in conjunction with earlier work, it follows that the ratio of the specific activity of the total carbon in carboxyl-labelled (A) to that of methyl-labelled (A) should be

$$\frac{1{\cdot}02\times 8}{14}\Big/\left(\frac{(1{\cdot}00\times 6)+(0{\cdot}075\times 8)}{14}\right)=1{\cdot}24.$$

The ratio found by experiment (Table 2) is 1·19, in satisfactory agreement. If one of the carbons C-8, C-11, C-12 or C-14 were a methyl carbon the calculated ratio would be 0·95; if one of these carbons were not derived from acetate at all, the ratio would be 1·09. Neither of these alternatives agrees so well with experiment.

Similar calculations were applied to fragments (E) and (F). These are fragments of the second degradation, and the dilutions of methyl-labelled and carboxyl-labelled material are different, so that the ratios given in Table 3 are not directly applicable. However, the origin of all carbons in (E) is known: it has six methyl and four carboxyl carbons. If one again sets 1·00 as the specific activity of a methyl carbon in methyl-labelled fragment (E), then the relative activity of a carboxyl carbon in the same specimen should still be 0·075 [since (E) originated from the same specimen of biosynthetic cholesterol as did the fragments of the first degradation], but the relative activity of a carboxyl carbon in carboxyl-labelled (E) must be calculated from the observed values (Table 2) for total carbon in the two specimens of (E), and is found to be

$$\frac{(6\times 1{\cdot}00)+(4\times 0{\cdot}075)}{10}\times\frac{5{\cdot}1}{17{\cdot}8}\times\frac{10}{4}=0{\cdot}45.$$

Thus we have in (E) the proportions of Table 4.

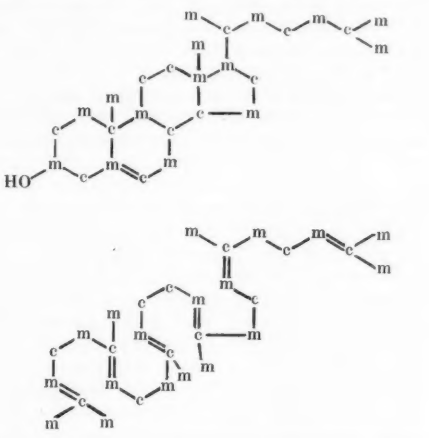


Table 3. *Relative specific activities of methyl and carboxyl carbons in methyl-labelled and carboxyl-labelled cholesterol, calculated from Table* 1

	Methyl-labelled cholesterol	Carboxyl-labelled cholesterol
Individual carboxyl carbon	0·075	1·02
Individual methyl carbon	1·00	0

Table 4. *Relative specific activities of methyl and carboxyl carbons in methyl-labelled and carboxyl-labelled fragment (E), calculated from Table* 2

	Methyl-labelled E	Carboxyl-labelled E
Individual carboxyl carbon	0·075	0·45
Individual methyl carbon	1·00	0

These results are now applicable to fragment (F), a product of the same degradations. If the evidence of electrolysis is valid, this contains nine methyl carbons and eight carboxyl carbons. The ratios of the specific activity of total carbon in methyl-labelled (F) to that in carboxyl-labelled (F) should then be

$$\frac{(9 \times 1{\cdot}00) + (8 \times 0{\cdot}075)}{17} \Big/ \left(\frac{(0{\cdot}45 \times 8)}{17}\right) = 2{\cdot}67.$$

The experimental value (Table 2) is 2·81. If (F) contained ten methyl carbons, the calculated ratio would be 3·34; if it contained nine methyl carbons, seven carboxyl carbons and one carbon not derived from acetic acid, the calculated ratio would be 3·02. Again the agreement is satisfactory if C-8, C-11, C-12 and C-14 are all carboxyl carbons, but is less satisfactory on other hypotheses. Earlier evidence (Little & Bloch, 1950; Würsch, Huang & Bloch, 1952; Cornforth *et al.* 1953*b*) indicating that cholesterol has 15 methyl and 12 carboxyl carbons supports the same conclusion.

The specimens of carbon dioxide obtained by electrolysis of (A) and (F) have 88% and 87% respectively of the specific activities calculated for carboxyl carbon in the parent acids; the carbon dioxide from C-9 has 95% of the specific activity calculated for methyl carbon. These slight but significant deviations from the calculated figures might be attributable to an isotope effect.

The completed pattern of methyl and carboxyl carbons in cholesterol is shown on p. 107 alongside the pattern previously discerned in squalene. The cyclization of squalene to cholesterol postulated by Woodward & Bloch (1953), whereby a methyl carbon migrates to C-13 to give a trimethylsteroid from which three methyl carbons are then eliminated, would lead to an arrangement of methyl and carboxyl carbons in cholesterol identical with that determined by experiment.

SUMMARY

1. Cholesterol biosynthesized by liver slices from [*carboxyl*-^{14}C] acetate and from [*Me*-^{14}C] acetate was chemically degraded by two diverging sequences of reactions.

2. From these two degradations, six fragments were obtained. The structure of one fragment was confirmed by synthesis.

3. From these fragments, one-carbon compounds originating from specified carbon atoms of cholesterol were prepared and their radioactivity was measured.

4. It is concluded that in cholesterol biosynthesized from acetate C-8, C-11, C-12, C-14 and C-16 originate from the carboxyl group of acetate and C-9, C-13, C-15, C-17 and C-18 originate from the methyl group.

5. The origin of all 27 carbon atoms in cholesterol biosynthesized from acetate is now known. The results are shown to harmonize with the current theory that squalene is a precursor of cholesterol in the animal body.

We wish to thank Glaxo Laboratories Ltd. for generous supplies of 7-dehydrocholesterol; Dr K. Miescher of CIBA for a gift of the Köster-Logemann tricyclic ketone; Dr A. T. James for liquid–vapour chromatography of some fatty acid mixtures; Mr G. Savage for the gas counting; and Mr J. Grant for technical assistance.

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The Metabolism of Short-chain Fatty Acids in the Sheep

5. SOME INTERRELATIONSHIPS IN THE METABOLISM OF FATTY ACIDS AND GLUCOSE BY SHEEP-RUMEN EPITHELIAL TISSUE*

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Acetic, propionic and butyric acids are produced in large quantities by microbial activity in the sheep rumen. Pennington (1952) showed that each of these acids may be metabolized by the rumen epithelial tissue, which produces ketone bodies from butyrate and, to a smaller extent, from acetate. Rumen epithelium also metabolizes glucose (Pennington & Sutherland, 1956*a*), which, although not normally present in appreciable quantity in the rumen, is available to the epithelial tissue from the blood.

It is of obvious physiological interest to study the metabolism of each one of these compounds in the presence of the others. Several workers have studied such interrelationships in other tissues. Quastel & Wheatley (1933) observed that propionic acid, but not glucose, lowered the production of acetoacetic acid from butyric acid by liver slices. Parnes & Wertheimer (1950) found that the utilization of glucose by rat diaphragm was decreased by acetate if the glucose concentration was not higher than 0·05%. Recent studies on the effects of glucose on the oxidation of fatty acids have given rather conflicting results. Allen, Friedmann & Weinhouse (1955) found that glucose had no appreciable effect on the oxidation of butyrate or palmitate by tissue slices and homogenates. However, by the use of a different technique, Lossow & Chaikoff (1955) observed that glucose spared the oxidation of palmitic acid but that the effect decreased with decrease in chain length of the acid.

* Part 4: Pennington & Sutherland (1956*b*).

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In the present work mixtures of these compounds were metabolized *in vitro* by rumen epithelium and the uptake of each substrate and the formation of ketone bodies were measured.

METHODS AND RESULTS

The procedures used in the preparation of the tissue sections, incubation of the tissue with the substrates and the method of ketone-body determination were as previously described (Pennington, 1952). The individual fatty acids in the mixtures were determined by gas–liquid chromatography (James & Martin, 1952). Glucose was determined by the method of Somogyi (1945). Tissue from a single sheep was used in each experiment. The sections were pooled before distributing among the flasks. The duplicate figures in Tables 1 and 3 represent the results obtained with different portions of tissue.

Table 1 shows the results obtained in two experiments in which all the possible combinations of acetate, propionate and butyrate were metabolized.

In spite of relatively large variations in the results, there is good evidence for some effects upon the metabolism of the fatty acids. The uptake of propionate was strongly inhibited by butyrate and that of butyrate inhibited by acetate; both effects were significant at the 1% level. The decrease in acetate uptake caused by propionate, the increase in acetate uptake in the presence of butyrate and the increased butyrate uptake when propionate was present were significant only at the 5% level.

The presence of propionate practically abolished ketone-body production from acetate but had only a small and possibly not significant (P approx. 0·1) effect on ketogenesis from butyrate.

Two unexpected effects were the apparent production of butyrate by the tissue when propionate and acetate were metabolized together, and the appearance of acetate when propionate and butyrate were used. The latter effect was amply confirmed, under various experimental conditions, by another experiment (Table 2).

The results obtained when each of the fatty acids was metabolized together with glucose are given in Table 3.

It is clear that glucose tended to increase the quantities of fatty acid metabolized. The uptake of glucose on the other hand, was, if anything, somewhat lowered in the presence of fatty acids. The

Table 1. *Metabolism of fatty acid mixtures by sheep-rumen epithelium*

The basal medium was Krebs–Ringer–bicarbonate (Cohen, 1945); 400 μmoles of each acid was added. Total vol., 20 ml. Wet wt. of tissue, 2 g. Incubated for 3 hr. at 39·5°. All results are expressed in μmoles. The ketone-body values are given as differences from the amounts produced by the tissue without added substrate (last line). A minus sign before an uptake figure denotes that the acid was actually produced.

Acid(s) added	Uptake of acids: Acetic	Propionic	Butyric	Ketone bodies produced
		Expt. 1		
Acetic, propionic, butyric	29, 82	24, −17	238, 185	194, 192
Acetic, propionic	17, 17	167, 143	−23·5, −12·5	1·2, 2·1
Acetic, butyric	93, 41	—	166, 190	200, 208
Acetic	47, 44	—	—	18, 18
Propionic, butyric	0, −36·5	31, 1	270, 307	209, 178
Propionic	—	149, 144	—	0·6, −1·7
Butyric	—	—	288, 248	224, 216
None	—	—	—	4·3, 4·8
		Expt. 2		
Acetic, propionic, butyric	2·7, −8	−58, −8	249, 232	209, 209
Acetic, propionic	−13, 18	144, 91	−23, −23	−2·8, −3·3
Acetic, butyric	92, 84	—	143, 127	235, 213
Acetic	18, 17	—	—	15·7, 12·5
Propionic, butyric	−40, −46	70, 29	214, 240	223, 204
Propionic	—	125, 71	—	−2·8, −3·3
Butyric	—	—	243, 217	235, 213
None	—	—	—	5·2, 6·7

Table 2. *Production of acetate by sheep-rumen epithelium*

Krebs–Ringer–bicarbonate (Cohen, 1945). Total vol., 20 ml. Temp., 39·5°. Quantities of fatty acids are expressed in μmoles.

Propionate added	Butyrate added	Wt. of tissue (g.)	Time (hr.)	Acetate produced
400	400	2	3	47
400	400	2	1½	27
400	400	4	3	72
400	400	2	6	39
400	100	2	3	31
100	400	2	3	46

Table 3. *Metabolism of glucose–fatty acid mixtures by sheep-rumen epithelium*

Krebs–Ringer–bicarbonate. Fatty acid, 100 μmoles; glucose, 111 μmoles. Total vol., 10 ml. Wet wt. of tissue, 2 g. Incubated for 3 hr. at 39·5°. All results are expressed in μmoles. The ketone-body values are given as differences from the amounts produced by the tissues without added substrate (last line).

Additions	Fatty acid uptake		Glucose uptake		Ketone bodies produced	
Acetate	6·2	7·7	—		1·3	3·2
Acetate + glucose	10·5	15·5	42·3	41·7	−1·9	−2·6
Propionate	37·7	40·2	—		−3·8	−3·1
Propionate + glucose	42·7	48·2	43·4	41·7	—	
Butyrate	71·2	73·0	—		57·8	56·6
Butyrate + glucose	74·2	78·7	35·6	38·3	52·3	48·7
Glucose	—		45·0	46·1	−3·7	−2·9
None	—		—		5·8	5·0

small formation of ketone bodies which accompanied the metabolism of acetate was sharply depressed by glucose, in spite of the increased uptake of acetate. The ketone-body production from butyrate was affected to a relatively much smaller extent by glucose.

DISCUSSION

Although the proportions may vary, all three acids used in this study are known to be present in the rumen, on a wide variety of natural diets. It is evident that a reliable quantitative estimate of the importance of the rumen epithelium in metabolizing the fatty acids produced in the rumen cannot be deduced from experiments with the single acids. In the experiments of Table 1, where equal amounts of the acids were present in the mixtures, the most definite effects were the inhibition of propionate uptake by butyrate and of butyrate uptake by acetate. In the normal rumen, with different proportions of the acids, other interrelationships may become more important.

Insufficient information is available to attempt to interpret the results. However, it does not seem that they can be explained by simple competition of the fatty acids for a common activating enzyme. The apparent interconversion of acetate and butyrate in the presence of propionate is of particular interest and merits further investigation. The effect of propionate and glucose upon ketone-body production from acetate accords with their known antiketogenic activity. However, the results suggest that, even in the presence of these compounds, butyrate will be an important source of ketone bodies in the sheep.

SUMMARY

1. Butyrate lowered the uptake of propionate by sheep-rumen epithelium, and acetate lowered the uptake of butyrate.

2. Ketone-body production from acetate was suppressed by propionate or glucose.

3. Some acetate was produced when propionate and butyrate were metabolized together.

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The Influence of Insulin on Free and Fixed Glycogen in the Diaphragm of the Rat

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Because of a difference in solubility in trichloroacetic acid, tissue glycogen can be divided into two forms, a free and a fixed form (Nerking, 1900; Willstätter & Rohdewald, 1934), recently called free and residual glycogen (Bloom, Lewis, Schumpert & Shen, 1951). The free glycogen is soluble in trichloroacetic acid at room temperature whereas the fixed fraction can be extracted only by heating the tissue for 15 min. at 100° in trichloroacetic acid (Kemp & Kits van Heijningen, 1954), or by destruction of the tissue in boiling potassium hydroxide. Previous investigations (Kits van Heijningen & Kemp, 1955) have made it unlikely that the fixation of glycogen to protein is an artifact, since extraction with an agent which does not precipitate proteins will yield the same values for both fractions, as does extraction with trichloroacetic acid. An investigation into the influence of muscular contraction on these fractions offered no clue as to their respective physiological functions (Kits van Heijningen & Kemp, 1955).

It is well established that insulin promotes the deposition of glycogen in striated muscle (Best, Dale, Hoet & Marks, 1926; cf. Gemmill, 1940). Should this increase in glycogen content be limited to one of these fractions, it might be argued that

that particular fraction serves primarily as a glycogen store. The incubation of rat diaphragm in a medium containing glucose with or without the addition of insulin, as described originally by Gemmill (1940), seemed particularly suitable to study the effect of insulin on the two glycogen fractions in muscle. Evidence will be presented that this increase in glycogen content is limited to the free fraction.

EXPERIMENTAL

Incubation media. The medium of Krebs & Henseleit (1932), buffered with phosphate to a pH of 7·3 and without $CaCl_2$ (Umbreit, 1951), was used. If necessary, the pH was adjusted to 7·3 after addition of insulin to the medium. To prevent precipitation of calcium phosphate, the $CaCl_2$ was added separately to the vessels after the tissue samples.

Insulin. In the first 13 experiments a commercial insulin solution (Novo Terapeutisk Laboratorium, Copenhagen, 40 i.u./ml.) was used; the other experiments were carried out with crystalline insulin (Philips-Roxane, Weesp, Netherlands, 24·3 i.u./mg.).

Manometer flasks. Dickens & Šimer (1930) flasks with a circular trough were used.

Methods. Female rats of a cross-bred Wistar strain, weighing 90–115 g., which had not been fasted, were anaesthetized with sodium pentobarbitone (60 mg./kg.) and bled from their carotid arteries. The diaphragms were removed immediately, care being taken to handle them as little as possible, to prevent contracture of the muscle. For the same reason preliminary soaking in cooled saline was abandoned, although this procedure would increase their glucose uptake (Brown, Park, Daughaday & Cornblath, 1952). The diaphragms were transferred immediately to oxygenated Krebs–Henseleit phosphate solution, pH 7·3, at 37±0·5°. The hemidiaphragms of one rat were placed in two separate tubes, and those of the next animal were added to the same tubes in reversed order so as to provide two comparable pairs of a left and a right hemidiaphragm in each tube. Four or six pairs were run at a time. When all the diaphragms had been removed, the pairs were taken from their storage tubes, blotted lightly on filter paper, weighed on a torsion balance, and differences in weight above 10 mg. between two corresponding pairs were corrected by cutting some tissue from the heavier pair. The pairs were transferred to manometer flasks containing 1·95 ml. of medium with 2 mg. of glucose/ml., with or without insulin, 0·05 ml. of 0·1 M-$CaCl_2$ being added after the tissue samples. Potassium hydroxide (1·8 N, 0·5 ml.) was placed in the surrounding trough. The final concentration of insulin was 0·87 i.u./ml. The vessels were gassed with O_2 for 3 min., equilibrated in the bath at 37·2° for 10 min. before readings were begun, and O_2 uptake was measured at 5 min. intervals for 60 min.

At the end of the incubation period the hemidiaphragms from each flask were blotted on filter paper, placed on top of one another and cut into two or three paired pieces, which

Table 1. *Influence of insulin on free-glycogen content of rat diaphragms incubated* in vitro

Pairs of hemidiaphragms were incubated for 60 min. in Krebs–Henseleit saline buffered with phosphate, containing 2 mg. of glucose/ml. Insulin, final concn. 0·87 i.u./ml., was added to one set, the other serving as a control. Insulin in Expts. 1–13 was Novo commercial insulin soln.; in Expts. 14–24 crystalline insulin (Philips–Roxane) was used. Values for free glycogen have been calculated as described by Kits van Heijningen & Kemp (1955).

	Control		Insulin-treated		Increase in free glycogen (g./100 g. wet wt.)
Expt. no.	Total wt. of pair and wt. of sample (mg.)	Free glycogen (g./100 g. wet wt.)	Total wt. of pair and wt. of sample (mg.)	Free glycogen (g./100 g. wet wt.)	
1	144 (96)	0·35, 0·34	144 (80)	0·41, 0·41	0·06
2	149 (122)	0·15, 0·16	150 (115)	0·40, 0·37	0·23
3	156 (114)	0·20, 0·19	159 (117)	0·37, 0·38	0·18
4	158 (108)	0·38, 0·38	164 (123)	0·42, 0·39	0·02
5	162 (111)	0·25, 0·25	165 (111)	0·42, 0·43	0·17
6	169 (131)	0·12, 0·12	165 (119)	0·41, 0·43	0·20
7	166 (121)	0·23, 0·22	176 (126)	0·40, 0·39	0·17
8	174 (138)	0·13, 0·13	184 (149)	0·31, 0·29	0·17
9	200 (148)	0·23, 0·22	203 (158)	0·39, 0·33	0·13
10	249 (211)	0·02, 0·02	250 (209)	0·12, 0·12	0·10
11	251 (208)	0·04, 0·04	256 (220)	0·22, 0·23	0·18
12	251 (214)	0·09, 0·09	259 (205)	0·08, 0·08	0·00
13	253 (220)	0·00, 0·00	263 (225)	0·27, 0·27	0·27
14	127 (74)	0·09, 0·10	129 (76)	0·52, 0·52	0·41
15	127 (70)	0·01, 0·02	139 (79)	0·31, 0·30	0·29
16	140 (78)	0·22, 0·24	135 (70)	0·44, 0·41	0·20
17	142 (82)	0·04, 0·05	140 (75)	0·44, 0·45	0·40
18	149 (81)	0·08, 0·10	142 (82)	0·52, 0·48	0·41
19	148 (78)	0·00, 0·00	156 (89)	0·18, 0·19	0·18
20	155 (79)	0·33, 0·31	157 (82)	0·50, 0·50	0·18
21	157 (87)	0·19, 0·18	158 (96)	0·47, 0·47	0·28
22	158 (88)	0·56, 0·55	159 (90)	0·66, 0·65	0·10
23	160 (93)	0·26, 0·24	167 (86)	0·50, 0·48	0·24
24	168 (86)	0·02, 0·00	167 (88)	0·20, 0·20	0·19
25	182 (107)	0·47, 0·46	173 (90)	0·55, 0·56	0·09

Table 2. *Fixed-glycogen content and* O_2 *uptake of the same pairs of hemidiaphragms as in Table* 1

	Control			Insulin-treated		
Expt. no.	Fixed glycogen (g./100 g. wet wt.)	O_2 uptake (μl./mg. wet wt./hr.)	O_2 uptake (μl./mg. dry wt./hr.)	Fixed glycogen (g./100 g. wet wt.)	O_2 uptake (μl./mg. wet wt./hr.)	O_2 uptake (μl./mg. dry wt./hr.)
1	0·19, 0·18	0·84	—	0·20, 0·20	0·85	—
2	0·13, 0·12	1·79	—	0·10, 0·11	1·65	—
3	0·16, 0·16	1·50	—	0·16, 0·17	1·43	—
4	0·18, 0·18	1·49	—	0·17, 0·17	1·55	—
5	0·14, 0·14	0·84	—	0·20, 0·17	0·80	—
6	0·17, 0·18	1·50	—	0·17, 0·17	1·47	—
7	0·16, 0·16	1·21	—	0·15, 0·16	1·31	—
8	0·09, 0·09	1·50	—	0·11, 0·10	1·37	—
9	0·21, 0·16	1·25	—	0·13, 0·14	1·17	—
10	0·07, 0·07	0·95	—	0·09, 0·09	0·95	—
11	0·10, 0·09	0·90	—	0·08, 0·08	0·85	—
12	0·07, 0·07	0·93	—	0·09, 0·09	0·95	—
13	0·09, 0·09	0·85	—	0·09, 0·09	0·86	—
14	0·21, 0·23	1·40	5·90	0·22, 0·22	1·38	5·95
15	0·20, 0·21	1·33	5·95	0·16, 0·16	1·25	5·25
16	0·14, 0·14	1·67	7·58	0·17, 0·19	1·77	7·18
17	0·17, 0·18	1·32	5·66	0·23, 0·22	1·44	6·55
18	0·17, 0·22	1·26	5·34	0·17, 0·18	1·26	5·30
19	0·16, 0·16	1·36	5·99	0·18, 0·18	1·35	5·80
20	0·30, 0·30	1·41	5·97	0·26, 0·25	1·34	5·32
21	0·14, 0·14	1·95	8·25	0·15, 0·13	1·75	7·30
22	0·26, 0·23	1·50	6·47	0·23, 0·23	1·47	6·62
23	0·23, 0·24	1·74	7·75	0·21, 0·21	1·65	7·10
24	0·21, 0·20	1·16	4·94	0·19, 0·20	1·26	5·58
25	0·21, 0·20	1·48	6·33	0·28, 0·28	1·54	6·65

were weighed on a torsion balance. One pair was transferred to 5 ml. of 80% (w/v) methanol to determine the free glucose and glucose 1-phosphate, another pair to 5 ml. of 5% (w/v) trichloroacetic acid for the determination of free and fixed glycogen, and, if present, the third pair was used to determine the dry weight. Free and fixed glycogen and the free glucose and glucose 1-phosphate concentrations were determined in duplicate in the trichloroacetic acid and methanol extracts by the method described in a previous paper (Kemp & Kits van Heijningen, 1954; cf. Mendel, Kemp & Myers, 1954).

RESULTS

In Table 1 the effect of insulin on the free-glycogen content of the diaphragms is shown. The pairs have been arranged in order of increasing weight. In the last 12 experiments the dry weight of a sample of each pair of hemidiaphragms was determined, and the O_2 consumption calculated/mg. dry wt. This was done to exclude any influence that insulin might have had on the wet weight of the tissues. In 22 out of these 25 experiments there was a rise in glycogen as compared with the controls, which was entirely accounted for by the increase in free glycogen. As shown in Table 2, there was also an increase in fixed-glycogen content in Expt. 25; in the control pair a very high free-glycogen content was found. In Expts. 4 and 12 there was no appreciable increase in glycogen content, only an increase in free glucose and glucose 1-phosphate (Table 3). The weight of the sample in Expt. 12 was 251 mg., one of the heaviest of the series.

The values for free glucose plus glucose 1-phosphate, and the difference between the insulin-treated pairs and their controls, are given in Table 3. In a number of rats of the same weight and sex as used in these experiments, free glucose plus glucose 1-phosphate was determined in the diaphragm immediately after killing the animals; these values are given in Table 4.

DISCUSSION

The effect of insulin on the glycogen content of the diaphragm of the rat appears to be limited to the free-glycogen fraction. In the only experiment which gave a rise in fixed glycogen as well, the free-glycogen concentration found in the control pair was one of the highest, not only of this series but of all values found in diaphragms tested so far. In this case the free fraction might have reached its maximum.

The oxygen uptake of the diaphragms was in agreement with the values reported by Stadie & Zapp (1947), but higher than those reported by Gemmill (1940), except for Expts. 10–13. In these experiments, however, the wet weight of the tissue per flask was higher than that used by Gemmill. In one of these four experiments the insulin-treated pair showed no increase in glycogen. It seems likely

Table 3. *Free glucose plus glucose 1-phosphate concentrations in the same pairs of hemidiaphragms as in Table 1*

	Control		Insulin-treated		
Expt. no.	Wt. of muscle sample (mg.)	Free glucose + glucose 1-phosphate (g./100 g. wet wt.)	Wt. of muscle sample (mg.)	Free glucose + glucose 1-phosphate (g./100 g. wet wt.)	Difference between controls and insulin-treated pairs
1	20	0·18, 0·20	33	0·22, 0·22	+0·03
2	27	0·12, 0·13	35	0·06, 0·11	−0·04
3	42	0·11	42	0·16	+0·05
4	50	0·03	41	0·07	+0·04
5	29	0·08, 0·14	45	0·20, 0·17	+0·07
6	38	0·16	46	0·09	−0·07
7	45	0·13	50	0·10	−0·03
8	36	0·11, 0·11	35	0·11	0·00
9	53	0·14	45	0·12, 0·11	−0·02
10	38	0·11	41	0·18	+0·07
11	43	0·17	36	0·18	+0·01
12	37	0·09	54	0·19	+0·10
13	33	0·20	38	0·15	−0·05
14	30	0·22, 0·19	31	0·15, 0·12	−0·07
15	27	0·27	27	0·18	−0·09
16	32	0·07, 0·07	28	0·08, 0·07	+0·01
17	24	0·15, 0·15	26	0·15, 0·15	0·00
18	38	0·15, 0·18	38	0·11, 0·16	−0·03
19	52	0·19, 0·20	38	0·17, 0·17	−0·03
20	42	0·27, 0·27	40	0·22, 0·22	−0·05
21	29	0·07, 0·11	32	0·04, 0·06	−0·04
22	37	0·12, 0·13	38	0·16, 0·17	+0·04
23	38	0·19, 0·17	50	0·20, 0·22	+0·03
24	37	0·20, 0·23	36	0·17, 0·20	−0·03
25	43	0·15, 0·15	41	0·19, 0·18	+0·04

Table 4. *Free glucose plus glucose 1-phosphate content of normal rat diaphragms*

Female rats, 80–115 g. body wt., were killed by bleeding under pentobarbitone anaesthesia. Mean values of three determinations are given ±S.E.

Rat no.	Wt. of samples (mg.)	Free glucose + glucose 1-phosphate (g./100 g. wet wt.)
1	28–41	0·13±0·01
2	28–56	0·13±0·03
3	28–43	0·13±0·04
4	31–37	0·13±0·03
5	36–60	0·11±0·03
6	57–60	0·10±0·03
7	51–70	0·22±0·04
8	47–76	0·19±0·03
9	49–72	0·22±0·02
10	39–42	0·19±0·03

that the comparatively low oxygen consumption in these experiments and the lack of increase in glycogen content in one of them is related to the greater weight and presumably greater thickness of these pairs, whereby the inner cells of the diaphragm might have taken no part in the metabolism (cf. Gemmill, 1940). This might also explain why the greatest increase in glycogen is found in the diaphragms with the lowest weight (see also Liébecq, 1954). For diaphragms of greater weight less insulin/mg. of tissue was available for fixation (Haugaard, Vaughan, Haugaard & Stadie, 1954) and this might have exerted an influence in the same direction.

Calculation of the oxygen consumption/mg. dry wt. showed no discrepancies compared with the consumption/mg. wet wt. which might have pointed to an influence of insulin on the wet weight of the tissue.

Since the animals were on ordinary laboratory diet, which contains about 60% of carbohydrate, and had access to food for 16 hr. a day, it was considered unlikely that insulin would cause a significant increase in oxygen consumption (Kerly & Ottaway, 1954). Therefore the agreement between oxygen consumption of insulin-treated pairs and their controls was used as a criterion for the similarity of circumstances. Pairs showing significant differences were rejected, though such differences did not materially alter the insulin effect.

The fixed glycogen remains remarkably constant in all experiments except in Expt. 25. It does not seem very likely therefore that the increase in free glycogen could have been derived from an initial increase in fixed glycogen, which would be abolished subsequently by transfer of an equal amount of glycogen from the fixed to the free fraction. A similar increase in free glycogen under the influence

of insulin is reported by Meyer, Russel, Platner, Purdy & Westfall (1955) in breis of rat's cardiac muscle. As pointed out by these authors, the normal intracellular relationship between the two fractions is disrupted in this case; this makes a quantitative transfer of the synthesized glycogen from the fixed to the free fraction even more unlikely. Therefore, direct storage of the extra glycogen produced under the influence of insulin in the free fraction seems at present the more simple explanation of the results obtained. In the beginning of a tetanic contraction Bloom & Knowlton (1953) demonstrated a decrease in free glycogen only. These authors (cf. Russell & Bloom, 1955) together with Meyer *et al.* (1955) take the view previously formulated by Przyłęçki & Majmin (1935), that it is the free fraction which takes part actively in the metabolism of glycogen, whereas the fixed fraction, which in their experiments remains more or less constant under various circumstances, would act as a depot. On the other hand, in experiments similar to those of Bloom & Knowlton (1953), but with less-frequent stimuli, either a decrease in both glycogen fractions or no effect at all has been obtained (Kits van Heijningen & Kemp, 1955). Neither in the experiments of Bloom nor in our own on contracting rat muscle could a transfer of glycogen from the free to the fixed form be excluded. Such a transition does not seem unlikely *a priori* if one assumes that one of these fractions acts as a depot. It has been demonstrated by Szent Györgyi (1951) that glycogen and myosin form an insoluble complex *in vitro*, which dissociates upon addition of adenosine triphosphate. A similar effect was obtained by the author with rat myosin and glycogen prepared from rat liver by extraction with trichloroacetic acid at room temperature. This does not, of course, prove that the fixed glycogen in intact muscle is also bound to myosin and released by adenosine triphosphate. Szent Györgyi (1951) assumes that this myosin–glycogen complex and its dissociation by adenosine triphosphate might play a part in the regulation of carbohydrate metabolism in muscle. Some light might be thrown on the sequence of events during normal muscular activity if it were possible to block the supposed transition from the free to the fixed form. From the present experiments it appears that conditions which promote glycogen deposition in muscle provoke an increase in free glycogen only.

SUMMARY

1. The effect of insulin on free and fixed glycogen of rat diaphragm has been investigated *in vitro*.

2. The increase in glycogen content brought about by insulin is limited to the free fraction.

3. The significance of this finding and the possible roles of both glycogen fractions in muscular contraction are discussed.

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The Content of Adenosine Polyphosphates in Fatty Livers

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It has been reported in a previous paper (Dianzani, 1954) that uncoupling of oxidative phosphorylation occurs in fatty livers. Adenosine triphosphatase (ATPase) activity of mitochondria isolated from fatty livers is increased. Conversely, many substances which inhibit oxidative phosphorylation produce fatty infiltration of the liver when administered intraperitoneally to rats or mice (Dianzani & Scuro, 1956). A decrease of adenosine triphosphate (ATP) and of phosphocreatine has been shown to occur in many organs of rats treated with 3:5-dinitro-*o*-cresol, a substance which inhibits oxidative phosphorylation (Parker, 1954). From these facts one may expect that ATP concentration in fatty liver is decreased.

Only scanty indirect data are available in the literature on organophosphorus compounds in fatty liver. Flock, Bollman & Mann (1936*a*, *b*) found a decrease of inorganic orthophosphate in the liver of dogs fed on a hyperlipidic diet, but reported a normal distribution of organophosphorus compounds in the liver of dogs treated with carbon tetrachloride. Kaplan & Greenberg (1944) observed a decrease of high-energy phosphates in fatty livers of rats fed on a hyperlipidic diet. Wagtendonk (1944) and Wagtendonk & Lamfrom (1945) described a marked decrease of ATP and phosphocreatine in liver, kidneys and muscles of rats fed on a diet deficient in vitamin E. It is well known that this deficiency produces fatty infiltration and necrosis in the liver, as well as muscular dystrophy (Himsworth, 1952). Ennor & Stocken (1948), on the contrary, found in the liver of guinea pigs treated with carbon tetrachloride a marked increase of those acid-soluble organophosphorus compounds having insoluble barium salts. This fraction contains mainly ATP, adenosine diphosphate (ADP) and hexose diphosphate. The results of Ennor & Stocken were confirmed by Rowles (1952), who used the enzymic method of Rowles & Stocken (1950) for the determination of ATP. The discrepancy in these results may be perhaps attributed to the different methods that have been used. Since new specific methods are now available for estimation of ATP, the problem of the content of ATP in fatty livers has been again investigated, and the results are described here.

MATERIAL AND METHODS

Production of fatty livers. Albino rats from an inbred strain, weighing 120–150 g., were used in most experiments. Guinea pigs weighing 250–300 g. were used in some instances. The animals were fed on a standard diet. Fatty infiltration of the liver was obtained by three different procedures: (1) by daily subcutaneous injection of CCl_4 [a 20% (v/v) solution in olive oil, 0·2 ml. for rats, 0·4 ml. for guinea pigs]; (2) by daily subcutaneous injection of a 0·5% solution of white phosphorus in olive oil (0·1 ml. for rats, 0·3 ml. for guinea pigs); (3) by feeding rats on a diet deficient in choline. The composition of this diet has been described in another paper (Dianzani, 1955). A control group of rats fed on the same diet received a daily dose of 5 mg. of choline given subcutaneously.

Preparation of homogenates and extracts. The animals were killed by decapitation in the cold room, and their organs (liver, kidney, heart and leg muscle) were immediately dissected out and weighed as quickly as possible. Homogenates (10%, w/v) were prepared with 0·25 M sucrose in a Potter–Elvehjem apparatus provided with a Lucite (polymethylmethacrylate, du Pont de Nemours) pestle. Cold trichloroacetic acid [0·1 ml. of 40% (w/v)] was immediately added. The whole procedure lasted approx. 30 sec. In some experiments, the organs were frozen with compressed gaseous CO_2 and then ground in a mortar chilled at −20°. Homogenization was then carried out in the usual way. When it was important to suspend the tissues in very small amounts of fluid the homogenization was done in a small mortar.

When mitochondria were to be isolated, 10% (w/v) homogenates in 0·25 M sucrose were first centrifuged at 1000 *g* for 10 min. in a Serval SS 1-Type angle centrifuge (Ivan Sorvall Inc., New York) placed in the cold room. The sediment was discarded and the mitochondria were then sedimented by centrifuging at 12 000 *g* for 30 min. They were used without any other washing.

ATP estimation. Four different methods (*A*, *B*, *C* and *D*) were employed for ATP estimation. Methods *A* and *B* were used with liver, kidney, heart and skeletal muscle; methods *C* and *D* were used only with liver.

Method *A* was the chemical fractionation procedure for phosphorus compounds in protein-free extracts, as described by LePage (1951). Deproteinization of homogenates was made by adding trichloroacetic acid. The mixture was filtered after standing for 10 min. in the cold room at 2°. The filtrate was neutralized with N-NaOH and then analysed for total phosphorus, 'apparent' inorganic phosphorus and 'true' inorganic phosphorus. This last was determined by adding to the neutralized filtrate 0·2 vol. of 10% (w/v) $CaCl_2$ saturated with $Ca(OH)_2$ at pH 8·8 (LePage, 1951). The

difference between the values for 'apparent' inorganic phosphorus and for 'true' inorganic phosphorus was taken to be the phosphorus in phosphocreatine. Fractionation of organic phosphates was made with barium acetate at pH 8·2. The fraction giving insoluble barium salts was analysed for acid-labile P (increase of inorganic phosphate after hydrolysis with N-HCl at 100° for 8 min.) and for ribose and fructose. This fraction contains mainly ATP and ADP, as well as hexose diphosphate. As hexose diphosphate was found to be hydrolysed to the extent of about 20% in N-HCl at 100° for 8 min., the amount of phosphate released from hexose diphosphate in each experiment was calculated from fructose estimations. These were made according to Roe (1934). The values obtained were then subtracted from those for acid-labile P in order to obtain the data for P released from ATP plus ADP. The relative concentrations of ATP and ADP were deduced from the value of the molar quotient acid-labile P/ribose. Ribose was determined by the method of Meijbaum (1939). All phosphorus determinations were made by the method of Berenblum & Chain (1938).

Method *B* was the enzymic procedure developed by Holloway (1954), which was adapted for tissue extracts. The enzymic reaction involved in this method is the conversion of 3-phosphoglycerate into 1:3-diphosphoglycerate in the presence of ATP and of a phosphoglycerate kinase from green peas. When 3-phosphoglycerate and the enzyme are in excess, the amount of 1:3-diphosphoglycerate which is formed is a measure of the amount of ATP present in the reaction medium. 1:3-Diphosphoglycerate is estimated by the hydroxamic acid assay described by Lipmann & Tuttle (1945) with some modifications introduced by Holloway (1954). Phosphoglycerate kinase was prepared according to Holloway (1954). The reaction medium contained ATP (potassium salt) or tissue extract, 0·056 M potassium 3-phosphoglycerate, 0·4 ml. of hydroxylamine reagent, 0·3 ml. of phosphoglycerate kinase and water to 2 ml. Incubation time was 2 hr. at 38°. Hydroxylamine reagent is 2·5 M hydroxylamine hydrochloride solution, pH 7·3, containing 0·015 M-$MgCl_2$. The reaction was stopped by the addition of 3 ml. of $FeCl_3$ reagent and 2·5 ml. of ethanol. The $FeCl_3$ reagent contained 8·3 g. of $FeCl_3, 6H_2O$, 0·20 g. of trichloroacetic acid and 42 ml. of conc. HCl made up to 500 ml. with water. The reaction mixture was then centrifuged at 3000 *g* before the optical density at 490 mμ. was read. The accuracy of the method was previously tested with different known amounts of ATP. Plotting the optical density against ATP concentration gave a straight line. The accuracy of the estimation was found to be $\pm 7\%$, within the range 0·2–2 μmoles of ATP. The reaction is specific for ATP. ADP, phosphocreatine and adenosine monophosphate, and different mixtures of these substances, gave negative results. As the sensitivity of the method is not high, large amounts of tissues must be used in order to prepare extracts for estimation of ATP. For this purpose, 1–2 g. of tissue was homogenized in a mortar with a small volume of water (3–4 ml.) containing 0·2 ml. of 40% (w/v) trichloroacetic acid. Precipitated proteins were discarded after centrifuging and the supernatant was neutralized with 30% (w/v) KOH. A portion (1 ml.) of the neutral fluid was used in each determination. Small amounts of substances giving positive colour reaction in the absence of the enzyme are present in tissue extracts, and a blank at zero time is therefore necessary. In some experiments, known amounts of ATP were added to the homogenate immediately before the addition of trichloroacetic acid, in order to study the extent of loss of ATP during the procedure. The recovery of added ATP was $95 \pm 6\%$.

Method *C* was the enzymic procedure recently described by Slater (1953). This method permits the separate estimation of hexose diphosphate, hexose monophosphate and high-energy phosphate ($\sim$P). It depends on the enzymic conversion of phosphorylated sugars into dihydroxyacetone phosphate, which then reacts with reduced diphosphopyridine nucleotide (DPN) in the presence of glycerol phosphate dehydrogenase. The amount of reduced DPN reacting is determined spectrophotometrically at 340 mμ. Slater's (1953) procedure *B* (*b*) measures the total $\sim$P content of an extract, i.e. ATP, ADP, phosphocreatine and phosphopyruvate, as well as phosphoglycerate. Slater's procedure *C* measures only ATP, so that from the two measurements it is possible to calculate the amount of ADP plus phosphocreatine plus phosphopyruvate plus phosphoglycerate by difference. Since phosphopyruvate and phosphoglycerate concentrations in liver tissue are usually low (LePage, 1951; Sacks, 1950; Albaum, Tepperman & Bodansky, 1946), one may assume that these values are due mainly to ADP plus phosphocreatine. Rabbit-muscle fractions *A* and *B*, necessary for the method, were prepared as described by Slater (1953). Hexokinase was a Sigma type II preparation from yeast (Sigma Chemical Co., 4648 Easton Avenue, St Louis, Missouri). Reduced DPN was obtained according to Ohlmeyer (1938). Tissue extracts were prepared from 10% (w/v) homogenates by addition of 0·5 ml. of 10% (w/v) trichloroacetic acid. The high sensitivity of this method permits the estimation of 0·01–0·02 μmole of ATP with considerable accuracy.

Method *D* was the chromatographic procedure developed by Eggleston & Hems (1952).

Adenosine triphosphatase was determined according to the method of Dubois & Potter (1943). Their reaction medium, however, was made isotonic by preparing all the solutions in 0·25 M sucrose.

Spectrophotometric determinations were made in a Beckman DU spectrophotometer.

Nitrogen was determined by the usual micro-Kjeldahl technique.

Total lipid content of the liver. This was determined by weighing the dry tissue powder before and after extraction with ether for 4 hr. in a Kumagawa apparatus. Drying of the liver to constant wt. was done in an oven at 85°.

Specimens for histological examination were prepared from each liver.

Reagents. ATP (sodium salt) of chromatographic-purity grade was obtained from Schwarz Lab. Inc., New York 17, N.Y. It gave only one spot in chromatograms. ATP (potassium salt) was prepared from the dibarium salt by solution of the latter in N-HCl, precipitation of barium with the calculated amount of H_2SO_4 and neutralization with 0·1 N-KOH. ADP (sodium salt) was prepared from the dibarium salt (Schwarz) by a similar procedure. Adenosine monophosphate (sodium salt) was prepared by neutralization of the acid (Schwarz). DPN of 90–95% purity was obtained from the Sigma Chemical Co. 3-Phosphoglycerate (potassium salt) was obtained from the dibarium salt (Sigma) by the same procedure as for the potassium salt of ATP. All other substances used were Merck (Darmstadt) products.

Table 1. *Content of some phosphorus fractions in liver, kidney, heart and skeletal muscle of normal rats and of rats with fatty infiltration of the liver*

Results are given as mg./g. wet wt. ± standard deviation.

Treatment	Organ	Total P	'True' inorganic P	Phospho-creatine P	Acid-labile P	Ribose in previous fraction	Molar quotient acid-labile P/ribose	Nitrogen	Total lipids
None (7 rats)	Liver	1·030 ± 0·030	0·311 ± 0·089	0·052 ± 0·013	0·241 ± 0·042	0·639 ± 0·117	1·82 ± 0·05	28·3 ± 2·5	36·4 ± 3·8
	Kidney	0·980 ± 0·090	0·387 ± 0·177	0·046 ± 0·019	0·114 ± 0·025	0·292 ± 0·101	1·88 ± 0·07	27·0 ± 2·2	—
	Heart	1·050 ± 0·071	0·578 ± 0·173	0·052 ± 0·011	0·115 ± 0·018	0·297 ± 0·039	1·87 ± 0·09	27·6 ± 1·9	—
	Muscle	1·290 ± 0·268	0·751 ± 0·168	0·049 ± 0·019	0·206 ± 0·026	0·554 ± 0·073	1·80 ± 0·03	27·1 ± 0·8	—
One injection of CCl_4 (6 rats)	Liver	0·960 ± 0·070	0·240 ± 0·060	0·036 ± 0·013	0·160 ± 0·020	0·500 ± 0·060	1·54 ± 0·02	28·0 ± 1·4	42·6 ± 2·5
	Kidney	0·990 ± 0·100	0·360 ± 0·120	0·039 ± 0·013	0·100 ± 0·020	0·285 ± 0·070	1·69 ± 0·04	27·0 ± 0·5	—
	Heart	1·060 ± 0·020	0·575 ± 0·090	0·048 ± 0·010	0·120 ± 0·020	0·300 ± 0·040	1·93 ± 0·15	26·7 ± 1·8	—
	Muscle	1·296 ± 0·220	0·750 ± 0·120	0·040 ± 0·020	0·210 ± 0·030	0·556 ± 0·070	1·82 ± 0·10	26·1 ± 0·7	—
Six injections of CCl_4 (6 rats)	Liver	0·954 ± 0·077	0·245 ± 0·053	0·024 ± 0·008	0·121 ± 0·051	0·470 ± 0·216	1·25 ± 0·11	25·0 ± 1·2	76·8 ± 6·9
	Kidney	0·976 ± 0·085	0·326 ± 0·060	0·026 ± 0·018	0·078 ± 0·016	0·242 ± 0·036	1·55 ± 0·12	26·8 ± 0·5	—
	Heart	1·073 ± 0·019	0·519 ± 0·096	0·040 ± 0·012	0·101 ± 0·019	0·279 ± 0·060	1·74 ± 0·11	27·2 ± 0·9	—
	Muscle	1·427 ± 0·127	0·806 ± 0·061	0·045 ± 0·012	0·188 ± 0·039	0·536 ± 0·135	1·69 ± 0·08	26·9 ± 1·9	—
One injection of white phosphorus (6 rats)	Liver	0·960 ± 0·070	0·240 ± 0·060	0·036 ± 0·013	0·160 ± 0·020	0·500 ± 0·060	1·54 ± 0·02	28·0 ± 1·4	—
	Kidney	0·990 ± 0·100	0·360 ± 0·120	0·039 ± 0·013	0·100 ± 0·020	0·285 ± 0·070	1·69 ± 0·04	27·0 ± 0·5	—
	Heart	1·060 ± 0·020	0·575 ± 0·090	0·048 ± 0·010	0·120 ± 0·020	0·300 ± 0·040	1·93 ± 0·15	27·5 ± 0·8	—
	Muscle	1·296 ± 0·220	0·750 ± 0·120	0·040 ± 0·020	0·210 ± 0·030	0·556 ± 0·070	1·82 ± 0·10	26·1 ± 0·7	—
Three injections of white phosphorus (6 rats)	Liver	0·901 ± 0·102	0·210 ± 0·057	0·029 ± 0·015	0·095 ± 0·041	0·372 ± 0·129	1·23 ± 0·10	25·8 ± 0·7	74·9 ± 5·7
	Kidney	0·899 ± 0·100	0·260 ± 0·032	0·039 ± 0·013	0·085 ± 0·018	0·284 ± 0·011	1·45 ± 0·19	26·9 ± 0·2	—
	Heart	1·079 ± 0·078	0·473 ± 0·083	0·045 ± 0·009	0·093 ± 0·014	0·261 ± 0·043	1·73 ± 0·05	26·9 ± 1·9	—
	Muscle	1·499 ± 0·074	0·734 ± 0·066	0·052 ± 0·014	0·225 ± 0·059	0·642 ± 0·183	1·70 ± 0·19	26·8 ± 0·1	—
Feeding on deficient diet for 1 week	Liver	0·935 ± 0·080	0·210 ± 0·057	0·022 ± 0·020	0·126 ± 0·020	0·390 ± 0·050	1·54 ± 0·10	29·0 ± 0·8	46·9 ± 1·2
	Kidney	0·930 ± 0·104	0·260 ± 0·032	0·038 ± 0·015	0·116 ± 0·025	0·315 ± 0·027	1·78 ± 0·15	28·0 ± 1·2	—
	Heart	1·219 ± 0·114	0·473 ± 0·083	0·041 ± 0·001	0·121 ± 0·010	0·340 ± 0·028	1·80 ± 0·03	27·6 ± 0·9	—
	Muscle	1·260 ± 0·029	0·734 ± 0·066	0·036 ± 0·004	0·195 ± 0·037	0·520 ± 0·076	1·82 ± 0·10	26·6 ± 0·9	—
Feeding on deficient diet for 6 weeks	Liver	0·891 ± 0·029	0·315 ± 0·074	0·013 ± 0·001	0·072 ± 0·013	0·316 ± 0·056	1·09 ± 0·04	25·2 ± 0·8	69·6 ± 6·8
	Kidney	1·095 ± 0·102	0·360 ± 0·062	0·026 ± 0·007	0·094 ± 0·030	0·294 ± 0·121	1·59 ± 0·24	27·4 ± 0·6	—
	Heart	1·246 ± 0·088	0·678 ± 0·030	0·042 ± 0·007	0·141 ± 0·012	0·386 ± 0·037	1·78 ± 0·15	28·0 ± 1·7	—
	Muscle	1·483 ± 0·145	0·864 ± 0·163	0·043 ± 0·015	0·185 ± 0·031	0·515 ± 0·070	1·75 ± 0·15	26·7 ± 0·5	—
Feeding on deficient diet + choline for 6 weeks (6 rats)	Liver	1·080 ± 0·180	0·320 ± 0·071	0·054 ± 0·012	0·236 ± 0·025	0·620 ± 0·102	1·84 ± 0·12	29·0 ± 0·9	35·8 ± 0·7
	Kidney	0·970 ± 0·087	0·328 ± 0·087	0·043 ± 0·010	0·110 ± 0·043	0·320 ± 0·056	1·68 ± 0·15	27·0 ± 1·4	—
	Heart	1·250 ± 0·123	0·570 ± 0·076	0·045 ± 0·012	0·100 ± 0·030	0·270 ± 0·018	1·73 ± 0·14	28·0 ± 0·8	—
	Muscle	1·470 ± 0·200	0·700 ± 0·098	0·046 ± 0·010	0·230 ± 0·060	0·600 ± 0·120	1·85 ± 0·13	26·8 ± 1·2	—

Statistical evaluation of results. The values obtained were submitted to statistical analysis, the standard deviation and the Student's t test being calculated for each average. Only differences with a t value corresponding to a probability $P<0{\cdot}05$ were accepted as significant.

RESULTS

Adenosine triphosphate content of rat organs

The results of the investigation on some rat organs for ATP content are represented in Tables 1–3. Table 1 reports the results which were obtained by the method of chemical fractionation of phosphate compounds in tissue extracts (LePage, 1951). Table 2 reports results obtained by the enzymic procedure of Holloway (1954). Table 3 gives a comparative picture of the values obtained by four different methods for ATP and ADP in the liver of normal and of treated rats. It is interesting to note that the values for ATP content of normal organs found by these methods do not differ much, and that they lie within the range of the standard deviation. This proves the reliability of all the methods used. The values found for ATP agree with those reported by other workers (Wildman, Campbell & Bonner, 1949; Rapoport, 1945; Venkataraman, Venkataraman, Schulman & Greenberg, 1950; Kaplan & Greenberg, 1944; Albaum, Tepperman & Bodansky, 1946; Albaum, Pankin & Harvill, 1953; Proger, Decaneas & Schmidt, 1945). The values obtained for ADP were always lower than those found for ATP. The molar ratio ATP:ADP was nearly 3:1 in all tissues studied. This value agrees substantially with those found for rat liver by Albaum, Tepperman & Bodansky (1946), by Albaum, Pankin & Harvill (1953) and by Oliver (1953), but differs remarkably from those reported by LePage (1946) (1:17), by Ennor & Stocken (1948) (from 1:1 to 1:7), by Rapoport (1945) (1:2) and by Rowles (1952) (9:1).

It is clear from Tables 1–3 that there is a marked decrease of ATP in all the types of fatty livers studied, the decrease being shown by all four methods of estimation. Table 1 shows that phosphocreatine also is decreased in fatty livers. It was found by the chemical fractionation method that the decrease of acid-labile P was not proportional to that of ribose contained in the same fraction. Calculation of relative amounts of ATP and ADP present in the fraction with insoluble barium salts, on the basis of the molar quotient acid-labile P/ribose, showed that the decrease of acid-labile P in fatty livers is due mainly to loss of ATP, whereas the ADP concentration increases significantly. This was confirmed by the data obtained by method *C*, which is an enzymic one. The molar ratio ATP:ADP, which was about 3:1 in normal livers, attained the value 1:15 in the livers of rats fed for six weeks on a diet deficient in choline (method *A*).

Table 2. *ATP content of some organs from normal and from treated rats, as determined by method* B *(see text)*

Values are given as mg./g. wet wt. ± standard deviation.

Treatment	No. of expts.	Liver		Kidney		Heart		Skeletal muscle	
		Nitrogen	ATP	Nitrogen	ATP	Nitrogen	ATP	Nitrogen	ATP
None	10	28·3 ± 0·7	0·922 ± 0·122	26·3 ± 0·5	0·456 ± 0·091	27·4 ± 4·8	0·454 ± 0·092	24·7 ± 0·8	0·743 ± 0·114
One injection of CCl_4	6	26·4 ± 0·6	0·600 ± 0·065	26·5 ± 0·3	0·470 ± 0·035	27·0 ± 2·3	0·500 ± 0·060	26·0 ± 0·5	0·820 ± 0·120
Six injections of CCl_4	8	26·2 ± 0·1	0·466 ± 0·051	25·1 ± 0·2	0·348 ± 0·113	26·9 ± 1·8	0·467 ± 0·136	25·4 ± 1·4	0·607 ± 0·105
One injection of white phosphorus	6	26·4 ± 0·6	0·620 ± 0·070	26·0 ± 0·1	0·420 ± 0·060	27·0 ± 1·6	0·490 ± 0·030	27·8 ± 0·3	0·800 ± 0·125
Three injections of white phosphorus	11	25·8 ± 1·8	0·393 ± 0·218	27·1 ± 2·0	0·602 ± 0·322	26·5 ± 2·0	0·608 ± 0·099	25·9 ± 1·6	0·600 ± 0·264
Feeding on deficient diet for 1 week	6	26·9 ± 2·1	0·670 ± 0·124	28·0 ± 1·5	0·396 ± 0·082	28·0 ± 1·2	0·550 ± 0·094	27·0 ± 1·1	0·910 ± 0·052
Feeding on deficient diet for 5 weeks	7	24·3 ± 3·6	0·240 ± 0·114	27·4 ± 1·4	0·296 ± 0·123	26·8 ± 1·4	0·608 ± 0·058	27·7 ± 1·9	0·914 ± 0·228
Feeding on deficient diet for 5 weeks and treatment with choline	6	27·5 ± 2·5	0·900 ± 0·070	27·0 ± 1·5	0·478 ± 0·085	27·2 ± 2·4	0·520 ± 0·060	27·5 ± 2·4	0·900 ± 0·120

Table 3. *ATP and ADP content of the liver of normal rats and of rats with fatty infiltration of the liver. Comparison of the results obtained by four different methods*

Method *A* is the chemical procedure of LePage (1951). Data for ATP and ADP were calculated from those of acid-labile P and of the molar quotient acid-labile P/ribose. Method *B* is the enzymic method of Holloway (1954). Method *C* is the enzymic procedure of Slater (1953) and method *D* the chromatographic method of Eggleston & Hems (1952). The values are given as mg./g. of tissue (wet wt.)±standard deviation. The values reported under ADP for method *C* are to be referred to ADP + phosphocreatine + phosphopyruvate + phosphoglycerate.

Treatment	Method	Nitrogen	Lipids	ATP	ADP	No. of expts.
None	*A*	28·3±2·5	36·4±3·8	1·077±0·192	0·298±0·107	7
	B	28·3±0·7	34·2±1·5	0·922±0·122	—	10
	C	28·6±1·4	36·1±4·8	0·917±0·196	0·289±0·045	7
	D	28·4±1·0	35·4±2·6	0·940±0·110	0·240±0·070	6
One injection of CCl_4	*A*	28·0±1·6	42·6±2·5	0·534±0·075	0·530±0·065	6
	B	26·4±0·6	40·4±5·6	0·600±0·065	—	6
Six injections of CCl_4	*A*	25·0±1·2	76·8±6·9	0·165±0·079	0·624±0·077	6
	B	26·2±0·1	70·4±4·9	0·466±0·051	—	8
	C	25·6±2·6	63·1±6·5	0·384±0·171	0·445±0·057	6
	D	25·0±3·6	75·6±8·9	0·430±0·160	0·450±0·120	6
One injection of white phosphorus	*A*	28·4±1·4	45·7±2·9	0·471±0·080	0·506±0·070	6
	B	26·4±0·6	40·4±5·6	0·620±0·070	—	6
Three injections of white phosphorus	*A*	25·8±0·7	74·9±5·7	0·119±0·113	0·503±0·149	6
	B	25·8±1·8	82·8±9·6	0·393±0·218	—	11
	D	24·0±1·5	79·8±9·2	0·380±0·150	0·500±0·090	6
Feeding on diet deficient in choline for 1 week	*A*	29·0±0·8	46·9±1·2	0·371±0·040	0·399±0·040	6
	B	26·9±2·1	40·9±3·6	0·670±0·124	—	6
	C	27·5±0·4	44·7±2·4	0·490±0·070	0·360±0·025	6
	D	27·0±2·5	46·5±4·8	0·560±0·128	0·320±0·040	6
Feeding on diet deficient in choline for 5–6 weeks	*A*	25·2±0·8	69·6±6·8	0·035±0·020	0·451±0·079	6
	B	24·3±3·6	75·6±4·8	0·240±0·114	—	6
	C	25·0±1·2	76·0±8·6	0·383±0·114	0·417±0·054	6
	D	24·2±2·6	63·6±4·5	0·159±0·068	0·560±0·052	6
Feeding on diet deficient in choline for 5–6 weeks and parenteral administration of choline	*A*	29·0±0·9	35·8±0·7	1·080±0·040	0·260±0·039	6
	B	27·5±2·5	34·2±1·9	0·900±0·070	—	6
	C	27·6±1·5	38·1±2·4	0·851±0·058	0·256±0·026	6
	D	29·6±2·4	39·5±4·6	0·920±0·060	0·265±0·040	6

The extent of the decrease of ATP was particularly great in the livers of rats receiving six injections of carbon tetrachloride, or three injections of white phosphorus, and of those fed for six weeks on the deficient diet. It was, however, noticeable also in the livers of rats fed on this diet for only one week, as well as in the livers of rats which received only one injection of carbon tetrachloride or phosphorus. Since the total-lipid content of the livers of these rats was not significantly increased, one may assume that depletion of ATP occurs before fatty infiltration. Nitrogen content of fatty livers was decreased, but to a considerably lesser extent than ATP, so that the decrease of ATP was significant also on a nitrogen-content basis.

ATP content of other tissues (kidney, heart and skeletal muscle) remained unaffected during the first stages of the treatments. After longer treatments, however, ATP decreased in the kidney, but it remained unaffected in both heart and skeletal muscle. Histological specimens of the kidneys in which ATP was decreased showed regressive processes such as cloudy swelling, tubular atrophy, haemorrhagic changes. No histological damage was found during the early stages of treatment, when ATP content was normal. This means that decrease of ATP is not a feature peculiar to fatty livers only, but probably occurs also in other regressive processes. In fact, decrease of P:O ratios was found also in cloudy swelling (Dianzani, 1954; Fonnesu & Severi, private communication). Also ~P was found to be decreased in the kidneys of guinea pigs treated with diphtheria toxin; here again histological examination showed cloudy swelling (Fonnesu & Severi, 1953).

ATP content of guinea-pig liver

The experiments of Ennor & Stocken (1948) and those of Rowles (1952), who found an increase of ~P in the liver after carbon tetrachloride poisoning, were made on guinea pigs. It seemed possible therefore that the discrepancy in the data found for rats during the present investigation, and those reported by these workers, could be referred to a species difference. The experiments were therefore repeated with guinea pigs. The results, which are given in Table 4, show that a decrease of ATP was found also in the liver of guinea pigs treated with

carbon tetrachloride or with white phosphorus. The extent of this decrease was, however, higher for rats than for guinea pigs.

Adenosine phosphates of isolated mitochondria

In some experiments, the adenosine phosphates of isolated mitochondria were determined in both normal and fatty livers, by the chromatographic method of estimation. The results, which are reported in Table 5, show that the percentage distribution of adenosine phosphates in liver mitochondria from normal rats resembles strongly that found by Bartley & Davies (1954) for sheep kidney-cortex mitochondria. These authors noted that the relative proportions of these compounds are much nearer the equilibrium of myokinase than those found in whole homogenate, and suggested that this was due to the presence of myokinase within mitochondria. It is clear from Table 5 that the amount of adenosine nucleotides is decreased in mitochondria from fatty livers. All three phosphates are decreased, but ATP is affected more than ADP.

Loss of adenosine phosphates from mitochondria after incubation in hypotonic medium

The decrease of adenosine phosphates in mitochondria from fatty livers could perhaps be due to their release into the surrounding medium. This was found to be the case for cytochrome *c* (Dianzani & Viti, 1955) and for pyridine nucleotides (Dianzani, 1955), and also for ATP (Blaschko, Born, D'Iorio & Eade, 1956) with ox adrenal-medullary granules submitted to hypotonic swelling. To test this possibility, mitochondria isolated from both normal and fatty livers were suspended either in 0·25 M sucrose or in water. Potassium fluoride (0·01 M) was added to the suspension medium in order to inhibit ATPase, preliminary experiments having shown that this concentration inhibits the enzyme to an extent of about 60%. The suspensions were allowed to stand at 28° for 10 min. and were then centrifuged at 12 000 *g* for 30 min. at 0°. The sediment was suspended in the same volume of water, and 0·1 ml. of 10% (w/v) trichloroacetic acid was added to both resuspended sediment and supernatant. The adenosine phosphates of the protein-free neutralized fluids were then determined by paper chromatography. It was found (Table 5) that a portion of the adenosine nucleotides is displaced from mitochondria into the surrounding medium as a consequence of incubation. With normal mitochondria, this portion was relatively small when the incubation medium was isotonic sucrose; it was, however, much higher when the incubation medium was water. With mitochondria isolated from fatty

Table 4. *ATP and ADP contents of the liver of normal guinea pigs and of guinea pigs with fatty infiltration of the liver*

Values are given as mg./g. wet wt. ± standard deviation. Method *A* is the chemical-fractionation procedure developed by LePage (1951). Method *D* is the chromatographic procedure.

Treatment	No. of expts.	Method	Nitrogen	Lipids	ATP	ADP
None	6	*A*	32·6 ± 2·5	30·6 ± 2·5	0·610 ± 0·045	0·193 ± 0·050
		D	32·4 ± 1·4	31·4 ± 2·7	0·635 ± 0·037	0·200 ± 0·050
Six injections of CCl_4	4	*A*	27·0 ± 2·4	62·5 ± 3·4	0·410 ± 0·050	0·315 ± 0·060
		D	26·6 ± 1·9	66·4 ± 7·8	0·395 ± 0·065	0·325 ± 0·135
Three injections of white phosphorus	4	*A*	27·0 ± 1·9	70·4 ± 5·6	0·360 ± 0·110	0·347 ± 0·100
		D	28·6 ± 2·5	79·4 ± 6·4	0·420 ± 0·080	0·315 ± 0·059

Table 5. *Release of adenosine nucleotides from mitochondria of normal and of fatty livers of rats after suspension in either isotonic or hypotonic medium*

Values are given as mg./10 g. of wet liver ± standard deviation. The chromatographic method was used and the number of the experiments was six in each case. Fatty infiltration of the liver was obtained by feeding rats on the diet deficient in choline for 4 weeks. AMP, adenosine monophosphate.

Source of mitochondria	Type of adenosine nucleotide	Nucleotide content before incubation	Nucleotide content after incubation in 0·25 M sucrose			Nucleotide content after incubation in water		
			Sediment	Supernatant	Recovery (%)	Sediment	Supernatant	Recovery (%)
Normal livers	ATP	0·800 ± 0·090	0·600 ± 0·070	0·100 ± 0·020	87·5	0·350 ± 0·090	0·200 ± 0·080	68·7
	ADP	0·980 ± 0·120	0·900 ± 0·060	0·160 ± 0·050	108·2	0·570 ± 0·065	0·560 ± 0·070	115·3
	AMP	1·300 ± 0·220	1·120 ± 0·140	0·200 ± 0·060	101·5	0·640 ± 0·110	0·690 ± 0·115	102·3
Fatty livers	ATP	0·180 ± 0·100	0·060 ± 0·050	0·060 ± 0·040	66·6	0·055 ± 0·010	0·060 ± 0·018	63·8
	ADP	0·520 ± 0·120	0·315 ± 0·068	0·290 ± 0·072	116·3	0·300 ± 0·075	0·310 ± 0·072	117·3
	AMP	0·650 ± 0·180	0·290 ± 0·075	0·340 ± 0·069	96·9	0·300 ± 0·090	0·345 ± 0·080	99·2

livers, the amount of adenosine nucleotides released into the surrounding medium was high, regardless of tonicity. Percentage recovery of ATP, calculated from the sum of ATP found in the sediment plus that found in the supernatant, was higher with normal mitochondria incubated with isotonic sucrose, than with the same mitochondria incubated with water. The reverse occurred for ADP. This means that a portion of ATP is destroyed through ATPase action during the incubation time. Here again, the change of incubation medium did not alter the behaviour of mitochondria from fatty liver.

DISCUSSION

This investigation shows that a marked decrease of ATP occurs in fatty livers of both rats and guinea pigs. Since the fatty livers have been produced by three different types of treatment, it seems evident that the decrease of ATP is a feature of this type of regressive process. Since the ATPase activity of fatty livers is increased, the question arises whether the loss of ATP occurs *in vivo*, or whether it is produced after the death of the animal during the preparation of homogenates. As this process lasted only about 30 sec., this second hypothesis seemed improbable. Some experiments were made, however, in which 0·25 M sucrose containing 0·01 M potassium fluoride was used for the preparation of the homogenates, in order to inhibit ATPase. Other samples from the liver of the same rats were homogenized in 0·25 M sucrose as usual. No difference was found between the values obtained for ATP and ADP either in the presence or in the absence of 0·01 M potassium fluoride. The hypothesis that loss of ATP occurs during homogenization as a consequence of ATPase activation is then highly improbable.

The results described in this paper with regard to ~P content in fatty livers agree with those of Kaplan & Greenberg (1944), who found decrease of these substances in the liver of rats fed on a hyperlipidic diet, and with those of Wagtendonk (1944), who found a marked decrease of ATP and phosphocreatine in fatty livers of rats fed on a diet deficient in vitamin E. They do not confirm, however, the results obtained by Ennor & Stocken (1948) and by Rowles (1952) on guinea pigs treated with carbon tetrachloride. In fact, Ennor & Stocken found an increase of acid-labile P, a fraction which contains mainly ATP, ADP and hexose diphosphate. The values for normal content of acid-labile P in guinea-pig extracts obtained by these workers, are, however, very low in comparison with those reported by other authors. Ennor & Stocken obtained for normal guinea pigs 5·3 mg./100 g. of wet liver. Fonnesu & Severi (1953) found 14·0 ± 1·5 mg./100 g. of wet liver; Wagtendonk (1944) obtained a figure of 14·2 ± 0·9. The values reported by this last author refer to 15 min. hydrolysis with N sulphuric acid at 100°, whereas those reported by Ennor & Stocken and by Fonnesu & Severi refer to 7 min. with N hydrochloric acid. It seems, however, improbable that this increase in the hydrolysis time is entirely responsible for the difference between the values of Wagtendonk and those of Ennor & Stocken. In fact, these authors stated that the release of inorganic orthophosphate P from the fraction with insoluble barium salts increases by only 2·0 mg./100 g. during the interval 7–15 min. Rowles (1952), who made enzymic determinations by the apyrase method of Rowles & Stocken (1950), reported a value of 3·3 mg./g. of liver nitrogen for the acid-labile P of adenosine polyphosphates of normal guinea pigs. The values for nitrogen content in the liver of these animals are not given. If one assumes, however, that the value found in the present investigation (32·6 mg./g.) is the normal nitrogen content, the values reported by Rowles correspond to 10·8 mg./100 g. of wet tissue, a value which is much higher than that obtained by Ennor & Stocken. A possible reason for the low values found by these authors may be found in their method of preparing extracts, which included freezing of the liver with liquid air before homogenization. Freezing is known to produce damage to mitochondria (Williams, 1952; Porter, Deming, Wright & Scott, 1953; Witter, Cottone & Stotz, 1954) as well as loss of soluble coenzymes from them. ATP requirements for choline and citrate oxidations are increased in these mitochondria and ATPase activity is also increased (Witter, Cottone & Stotz, 1954). Some ATPase estimations which were made during the present investigation confirmed this. The ATPase activity of mitochondria isolated from non-frozen liver was found to be able to release from ATP 7·55 ± 0·31 mg. of inorganic P/hr./g. of wet liver, and the value for mitochondria isolated from frozen liver was 13·6 ± 0·8. (The number of experiments was six for each group.) The fact that Ennor & Stocken obtained by the chemical method of

Table 6. *Influence of previous freezing on ATP and ADP content of normal guinea-pig liver*

Values are given as mg./g. of wet liver.

Expt. no.	Method	Non-frozen liver ATP	Non-frozen liver ADP	Frozen liver ATP	Frozen liver ADP
1	*A*	0·635	0·200	0·375	0·280
	D	0·660	0·210	0·390	0·290
2	*A*	0·595	0·215	0·360	0·275
	D	0·610	0·210	0·360	0·260
3	*A*	0·620	0·198	0·355	0·242
	D	0·640	0·205	0·345	0·250

estimation ATP:ADP ratios ranging from 1:1 to 1:7, and that the ratio was 3:1 in the present experiments, strongly supports this interpretation. In other experiments by Ennor & Stocken, in which the relative amount of ATP and ADP were calculated by the myosin ATPase method, ATP:ADP ratios ranged from 90:1 to 2:1.

In some experiments which were made during the present investigation, extracts were prepared from frozen livers, freezing being obtained very quickly with compressed gaseous carbon dioxide. The ATP content of normal guinea-pig liver was found to be about 40% lower by this method than by the procedure consisting of direct homogenization without previous freezing. ADP was higher under the same conditions (Table 6). Lower values for adenosine polyphosphates in frozen than in non-frozen livers were observed also by Rowles (1952). If this explanation for the low values reported by Ennor & Stocken is valid, one may perhaps explain also why these authors found higher acid-labile P levels in fatty than in normal livers. In fact, fatty livers contain much more ADP than the normal ones; they must then be affected by ATPase activation to a much lesser degree. The increase of ADP in fatty livers may be due either to increased rate of synthesis or to decreased disappearance. Since ATPase is increased and oxidative phosphorylation is inhibited, whereas the myokinase reaction is quite normal, it seems probable that all these phenomena contribute to the increase of ADP.

The decrease of ATP is particularly marked in mitochondria. Also ADP and adenosine monophosphate are decreased in these particles. One of the reasons for this decrease seems to be their displacement from the mitochondria into the surrounding medium. This was found to happen also with cytochrome *c* and the pyridine nucleotides. Loss of adenosine nucleotides was observed also with normal mitochondria suspended in water; it seems probable that it is concerned with swelling of mitochondria and with their increased permeability. Loss of soluble coenzymes and cofactors seems then to be a rather general feature of swollen mitochondria. In fatty livers, the decrease of these factors (ATP and DPN) seems to occur before the beginning of fatty infiltration; it is then probable that this phenomenon is responsible for accumulation of fat. In fact, ATP is necessary for the activation of fatty acid oxidation, and DPN is an obligatory hydrogen acceptor in the fatty acid cycle (Lipmann, Jones, Black & Flynn, 1952; Lynen & Ochoa (1953).

SUMMARY

1. The content of adenosine phosphates in liver, kidney, heart and skeletal muscle has been studied by four different methods in normal rats and in rats with fatty livers. The fatty infiltration of the liver was obtained by three different procedures (treatment with carbon tetrachloride; treatment with white phosphorus; feeding on a diet deficient in choline).

2. A marked decrease of adenosine triphosphate (ATP) was found in fatty livers by all four methods of estimation. Adenosine diphosphate (ADP) was increased.

3. Decrease of ATP and increase of ADP occurred in the livers of treated animals before the beginning of fatty infiltration.

4. ATP decreased slightly also in the kidneys of treated animals, but only after long treatment. Histological examination of these kidneys showed cloudy swelling, tubular atrophy, haemorrhagic changes. No change was found in the ATP content in the heart or skeletal muscle.

5. The concentration of adenosine phosphates was much decreased in mitochondria isolated from fatty livers. Mitochondria isolated from normal livers released more adenosine phosphates into the surrounding medium when they were suspended in water than when in isotonic sucrose. Mitochondria from fatty livers released about the same amount of adenosine phosphates both in hypotonic and in isotonic media.

6. Possible explanations of the experimental results are discussed.

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The Transformation of Purines into Pteridines

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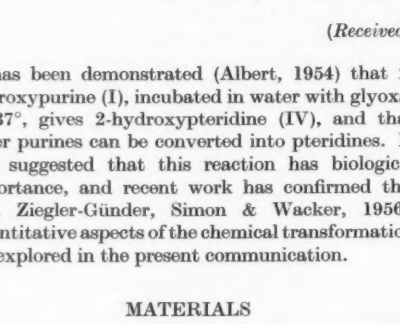
It has been demonstrated (Albert, 1954) that 2-hydroxypurine (I), incubated in water with glyoxal at 37°, gives 2-hydroxypteridine (IV), and that other purines can be converted into pteridines. It was suggested that this reaction has biological importance, and recent work has confirmed this (e.g. Ziegler-Günder, Simon & Wacker, 1956). Quantitative aspects of the chemical transformation are explored in the present communication.

MATERIALS

2-Hydroxypurine was obtained by the action of nitrous acid on 2-aminopurine (Albert & Brown, 1954), 2-hydroxy-9-methylpurine by the action of formic acid on 5-amino-2-hydroxy-4-methylaminopyrimidine (Brown & Mason, 1957) and 9-methylpurine by the action of formic acid on 5-amino-4-methylaminopyrimidine (Albert & Brown, 1954).



Hypoxanthine, xanthine and guanine were commercial preparations recrystallized to chromatographic purity and maximal absorption at $\lambda_{max.}$ (see Mason, 1954).

2-Hydroxy- (Albert, Brown & Cheeseman, 1951), 2:6-dihydroxy- (Albert, Lister & Pedersen, 1956), 4:6-dihydroxy- and 4:6:7-trihydroxy-pteridine (Albert & Brown, 1953) and xanthopterin (Albert & Wood, 1952) were made by standard methods. 6:7:8-Trimethyl-2-pteridone was kindly prepared by Dr D. J. Brown, from diacetyl and 5-amino-2-hydroxy-4-methylaminopyrimidine (Brown & Mason, 1957). Ethyl glyoxylate hemiacetal was prepared from ethyl tartrate (Rigby, 1950). Glyoxylic acid (crystalline) and glyoxal (50% syrup) were purchased. 4:5-Diamino-2-hydroxypyrimidine was prepared as by Johns (1911).

A Hilger Uvispek spectrophotometer with 4 cm. cells was used.

EXPERIMENTAL

2-Hydroxypurine to 2-hydroxypteridine. 2-Hydroxypurine monohydrate (0·46 g.; 0·003 mole), glyoxal (1·38 g. of 50% syrup; 0·012 mole) and water (6·5 ml.) were set aside at 37° for a week, then at 0° for 3 days (final pH was 2). The solid, recrystallized from 50 parts of boiling water (with charcoal), gave a yield of 16% of 2-hydroxypteridine monohydrate. This was chromatographically [on paper, in *n*-butanol–5N acetic acid (7:3), also in 3% (w/v) aqueous NH_4Cl] and spectrometrically identical with authentic 2-hydroxypteridine. (Found for material dried at 120°/0·1 mm.: C, 43·6; H, 3·8; N, 33·3. Calc. for $C_6H_4ON_4,H_2O$: C, 43·4; H, 3·6; N, 33·7%.)

From the same quantities of materials plus 5N-H_2SO_4 (1·8 ml.) set aside at 37° for 24 hr. (final pH, 0·5), 0·26 g. (52%) of pure 2-hydroxypteridine monohydrate was isolated; but 84% was demonstrated to have been formed, by spectrometric examination (pH 7) of the neutral molecule at 230 and 307 mμ. When the incubation was prolonged for a week, or when a higher temperature was used, the

yield fell to 39%, and a purple substance, insoluble in boiling water, was also formed.

4:5-Diamino-2-hydroxypyrimidine to 2-hydroxypteridine. Glyoxal (1·38 ml. of 50% syrup) in water (7 ml.) was adjusted to pH 7 with 2 N-Na_2CO_3. 4:5-Diamino-2-hydroxypyrimidine (0·38 g.) was added, and the solution set aside at 37° for 24 hr. and refrigerated (final pH, 6·5). 2-Hydroxypteridine monohydrate (0·49 g.; 93%) was deposited as colourless crystals, chromatographically and spectroscopically pure.

2-Hydroxypurine to 2:6-dihydroxypteridine. 2-Hydroxypurine monohydrate (0·154 g.) was dissolved in boiling water (1·5 ml.) and glyoxylic acid monohydrate (0·28 g.) added. The solution was cooled and 2·5 N-HCl (1·2 ml.) added. The mixture was set aside at 37° for 72 hr. Without filtration, the precipitate was dissolved in N-KOH (8 ml.) and diluted to 50 ml. with water. Spectrometric examination at 412 mμ. after dilution showed that 84·6% of 2:6-dihydroxypteridine had been formed. (2-Hydroxypurine has $\lambda_{max.}$ 265 in 0·1 N-KOH.)

The substitution of ethyl glyoxylate hemiacetal (0·3 g.) for glyoxylic acid lowered the yield to 78·8%. In this instance the remainder of the alkaline solution was adjusted to pH 4·9 at 80° with 0·1 N acetic acid (25 ml.) and 0·1 N-HCl. The mixture, refrigerated for 4 days, gave 2:6-dihydroxypteridine (0·1267 g.; 71%). Identity was established by infrared and ultraviolet spectroscopy, and by chromatography. 2:7-Dihydroxypteridine could not be detected, but this isomer predominates at pH values above 5 (Albert *et al.* 1956).

Hypoxanthine to 4:6-dihydroxypteridine. Hypoxanthine (0·034 g.), ethyl glyoxylate hemiacetal (0·074 g.) and N-HCl (0·75 ml.) were heated in a sealed tube at 120° for 6 hr. N Potassium hydroxide (1·5 ml.) was added and the solution was diluted to 5 ml. A control solution (0·001 M) of 4:6-dihydroxypteridine monohydrate was made in 0·01 N-KOH.

Two bands, each about 8 cm. wide, of the reaction solution (0·1 ml.) were applied to each of two sheets of Whatman no. 1 paper. The control solution (0·1 ml.) was similarly applied to a third paper. The three papers were run as ascending chromatograms in 2 N-NH_3 soln. The papers were dried, examined in ultraviolet light at 365 mμ. and then as rapidly as possible at 254 mμ.

The following strips (12 cm. × 3 cm.) were cut: (*a*) four containing the 4:6-dihydroxypteridine formed in the reaction; (*b*) one containing the 4:6-dihydroxypteridine control and (*c*) one of blank paper from the same R_F location. These strips were eluted overnight with 0·1 N-KOH. The eluates from (*a*) were combined and all eluates were diluted to 10 ml. with 0·1 N-KOH: the optical densities of eluates (*a*) and (*b*) were measured at 367 mμ. with the eluate (*c*) as blank. The recovery from the control was 100%. The yield of 4:6-dihydroxypteridine formed from hypoxanthine was 0·49%.

Hydrolysis of guanine to xanthine. Guanine (0·076 g.) and N-HCl (1·5 ml.) were heated for 6 hr. in a sealed tube, chromatographed in 3% (w/v) NH_4Cl and eluted with 0·1 N-KOH as above. At 120° 82% of guanine (measured at 274 mμ.) was unchanged and 12% was converted into xanthine (measured at 283 mμ.). The corresponding percentages at 150° were 20 and 47.

Guanine to xanthopterin. Guanine (0·0378 g.), ethyl glyoxylate hemiacetal (0·074 g.) and N-HCl (0·75 ml.) were heated in a sealed tube for 6 hr. at 110°. The yellow solution was cooled, N-KOH added (1·5 ml.) and made up to 5 ml. with water (solution is 5×10^{-2} M with respect to guanine). Control solutions were made from xanthopterin monohydrate (1·97 mg.) and 2:4:6-trihydroxypteridine (1·8 mg.) in 0·1 N-KOH (10 ml.).

Paper chromatograms were run in a similar way to those for hypoxanthine. The recovery of control xanthopterin and 2:4:6-trihydroxypterin was 99·6 and 102·4% respectively. The yield of these compounds formed from guanine was 0·08 and 0·26%. Measurements were made at 390 mμ. for both substances.

2-Hydroxy-9-methylpurine to 6:7:8-trimethyl-2-pteridone. 2-Hydroxy-9-methylpurine (0·0375 g.), diacetyl (0·086 g.) and 0·01 N-HCl (37·5 ml.) were set aside at 37° for 72 hr. To the yellow solution (black if 0·1 N acid was substituted) 0·1 M-KH_2PO_4 soln. (7·5 ml.) was added, the pH was adjusted to 7·0 with N-KOH, and the solution was diluted to 50 ml. A control solution was made from 6:7:8-trimethyl-2-pteridone (9·5 mg.) in water (25 ml.).

The reaction solution and the control (0·1 ml. of each) were applied as above to paper, which was developed in 3% NH_4Cl. The excised bands were eluted with water, and each eluate was diluted to 10 ml. with water. Spectrophotometry at 327 mμ. showed that 65% of the control had been destroyed in the processing, and a new peak developed at 298 mμ. (apparently 5-amino-2-hydroxy-4-methylaminopyrimidine). If a similar rate of hydrolysis is assumed the yield from 2-hydroxy-9-methylpurine was approximately 7%.

RESULTS

The principal results of these transformations are summarized in Table 1. In several experiments, paper chromatography followed by elution and spectrophotometry was used because of the small yields. It is noteworthy that 2 N-NH_3 soln. had no effect upon 4:6-dihydroxypteridine and xanthopterin, whereas it rapidly forms a secondary amine with 6-hydroxypteridine, even at 20° (Albert, 1955).

DISCUSSION

The conversion of purines into pterodines has no preparative value (because both are made from the same intermediates), but is of interest for the light which it may shed on the biological origin of natural pteridines. Provided that a purine is suitably substituted, the imidazole ring opens readily. For example, 2-hydroxypurine (I) gives 4-amino-5-formamido-2-hydroxypyrimidine (II) at 20° and pH 5 (2-mercaptopurine behaves similarly) (Albert & Brown, 1954). The formyl derivative (II) loses the formyl group more and more rapidly as it becomes autocatalysed by H^+ ions from the liberated formic acid, giving 4:5-diamino-2-hydroxypyrimidine (III) (Albert & Brown, 1954). It is shown in the Experimental section that 4:5-diamino-2-hydroxypyrimidine combines with glyoxal to give 2-hydroxypteridine (IV) in 93% yield at 37° and pH 7. This is typical of a general reaction,

which proceeds under physiological conditions regardless of substituents in the pyrimidine and requires no catalyst. 1:2-Diketones, aldehydo acids and keto acids react similarly to glyoxal. The hydroxyl group, derived from aldehydo acids, goes to the 6 position of pteridines under acid (and perhaps enzymic) conditions, but sometimes to the 7 position when the pH is raised (Albert, Brown & Cheeseman, 1952).

In Table 1 the results cover these three consecutive reactions, i.e. the purine, two-carbon fragment and solvent were mixed and the yield of the pteridine was measured. Expt. 1 shows a yield of 16% of 2-hydroxypteridine from 2-hydroxypurine and glyoxal in water at 37°. The rate-limiting reaction is (II) → (III), so that (II) accumulated (Albert & Brown, 1954). A much better yield (84%) was obtained in Expt. 2, under more acid conditions. 2-Hydroxypteridine is itself sensitive to acid, and forms an insoluble purple substance, which appears to be a dimer (Albert *et al.* 1951): thus the yield fell on longer incubation (Expt. 3).

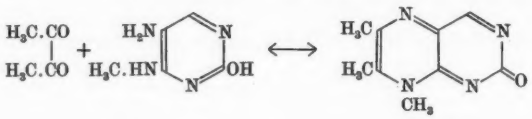
Dihydroxypteridines, however, are not affected by acid, and hence a longer incubation was given when glyoxylic acid and 2-hydroxypurine were combined, and a yield of 85% of 2:6-dihydroxypteridine resulted (Expt. 4). Ethyl glyoxylate hemiacetal gave similar results (Expt. 5) and, since it is more accessible than glyoxylic acid, was used in Expts. 6 and 7.

A hydroxyl group in the 6 (or 8) position of purine does not facilitate degradation to the same extent as in the 2 position. Even at 120°, hypoxanthine gave only 0·5% of 4:6-dihydroxypteridine (Expt. 6).

Since 12% of guanine was converted into xanthine at 120° in N hydrochloric acid in 6 hr. the reaction between guanine and ethyl glyoxylate was carried out at 110°. However, the reactivity of the degradation products of xanthine proved to be greater than those of guanine because more 2:4:6-trihydroxypteridine than the 2-amino-4:6-dihydroxy compound (xanthopterin) was produced, and the total yield was small (Expt. 7).

When the 9 position in a purine is alkylated, as in naturally occurring purines, considerations of valency lead to a different type of product with glyoxal and diketones. Thus to give a pteridine with diacetyl, 5-amino-2-hydroxy-4-methylaminopyrimidine (V), from the degradation of 2-hydroxy-9-methylpurine, must surrender a hydrogen from the hydroxyl group, and submit to a quinonoid rearrangement of bonds as in riboflavin. The product, 6:7:8-trimethyl-2-pteridone (VI) was obtained in only moderate yield (Expt. 8), because this arrangement of bonds gives a strained molecule in the pteridine series; in the presence of acid the reverse reaction, to (V) and diacetyl, also occurs.

It is interesting to consider the course that the transformation would follow in alkaline media.



(V) (VI)

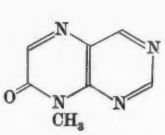
Table 1. *Transformation of purines into pteridines*

Expt. no.	Purine starting material	1:2-Dicarbonyl substance	Temp.	Solvent	Time (hr.)	Pteridine product	Yield (%)
1	2-Hydroxypurine	Glyoxal	37°	Water	168	2-Hydroxypteridine	16
2	2-Hydroxypurine	Glyoxal	37	N-H_2SO_4	24	2-Hydroxypteridine	84
3	2-Hydroxypurine	Glyoxal	37	N-H_2SO_4	168	2-Hydroxypteridine	39*
4	2-Hydroxypurine	Glyoxylic acid	37	N-HCl	72	2:6-Dihydroxypteridine	85
5	2-Hydroxypurine	Ethyl glyoxylate hemiacetal	37	N-HCl	72	2:6-Dihydroxypteridine	79
6	6-Hydroxypurine	Ethyl glyoxylate hemiacetal	120	N-HCl	6	4:6-Dihydroxypteridine	0·49
7	2-Amino-6-hydroxypurine	Ethyl glyoxylate hemiacetal	110	N-HCl	6	2-Amino-4:6-dihydroxypteridine [xanthopterin]	0·08
						2:4:6-Trihydroxypteridine	0·26
8	2-Hydroxy-9-methylpurine	Diacetyl	37	0·01 N-HCl	72	6:7:8-Trimethyl-2-pteridone	~7

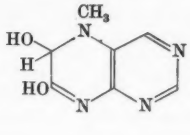
* Plus a quantity of the purple substance (? dimer) formed by the action of acid on 2-hydroxypteridine (Albert *et al.* 1951).

9-Methylpurine is easily split by alkali (to 5-amino-4-methylaminopyrimidine) (Albert & Brown, 1954), and so is 9-ribosylpurine (Brown, 1957). In general, those purines so substituted as to give no ionizable hydrogen undergo this decomposition, e.g. 7-methyladenine or 1:7-dimethylhypoxanthine. A very few purines, notably 9-methyladenine and adenosine, fulfil this structural requirement and yet are not split by boiling N sodium hydroxide. The choice of the suitable dicarbonyl substance for these alkaline condensations is limited. However, ethyl glyoxylate hemiacetal survived boiling 2N sodium carbonate for an hour, giving a yield of 77% of 7-hydroxypteridine from 4:5-diaminopyrimidine (Albert *et al.* 1956), and a little 8-methyl-7-pteridone (VII) was obtained from 9-methylpurine. The 1:2-diketones, although stable to alkali, introduce difficulties in satisfying valency.

The products from 7-methylpurines and glyoxylic acid should have quite another character, because Brown & Mason (1957) showed that 4-amino-5-methylaminopyrimidine and glyoxylic acid (except in strong acid) give a dihydropteridine, 5:6-dihydro-6:7-dihydroxy-5-methylpteridine (VIII).



(VII)



(VIII)

Biological implications. It has been suggested (Albert, 1954) that the biological origin of pteridines is from the union of naturally occurring 1:2-dicarbonyl substances (such as glyoxylic, pyruvic and oxaloacetic acids) and 4:5-diaminopyrimidines, and that these pyrimidines arise by the degradation of natural purines or purine ribosides. Some confirmation of these hypotheses has recently been published. [2-^{14}C]2:4:5-Triamino-6-hydroxypyrimidine was fed to pierid caterpillars; [2-^{14}C]xanthopterin was isolated from the wings (Weygand & Waldschmidt, 1955). More relevant is the transformation of a purine into a pteridine in larvae of the amphibian *Xenopus*. These were intradermally injected with [2-^{14}C]guanine, then ten days later the skins were extracted with N ammonia solution at 20° and chromatographed. A blue-fluorescing spot was oxidized to 2-amino-4-hydroxypteridine-6-carboxylic acid, which was radioactive (Ziegler-Günder *et al.* 1956).

The purine → pteridine reaction may be significant in the regulation of growth, because some pteridines have powerful and specific growth-regulating properties. For example, formyltetrahydropteroylglutamic acid is essential, in traces, for driving the mitosis of mammalian cells past metaphase into anaphase (Jacobson, 1952, 1954). Also, traces of xanthopterin enormously increase the mitotic rate in mammalian kidneys, which grow far beyond their normal size (Haddow, 1947, and personal communication). Although cells contain a comparatively large amount of purines, the amount of pteridines is usually minute. Hence it is conceivable that natural growth could be accelerated by facilitating reactions of the type (I) → (IV) through a local abundance of either 1:2-dicarbonyl substances or of the transforming enzyme(s).

SUMMARY

1. 2-Hydroxypurine reacts with 1:2-dicarbonyl substances, such as glyoxal or glyoxylic acid, to give the corresponding pteridines in good yield.

2. Guanine and hypoxanthine undergo similar transformations, but less readily.

3. The evidence that naturally occurring pteridines arise from purines (or purine derivatives) is reviewed, and it is suggested that this reaction may regulate growth in certain circumstances.

Mr E. P. Serjeant is thanked for the chromatographic analyses.

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A Colorimetric Method for the Determination of Aliphatic Amines in the Presence of Ammonia

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During studies on the formation of lower aliphatic amines by bacteria (King, 1953; Ekladius & King, 1956) a method was needed for estimating these compounds in culture filtrates. Alkaline distillation will separate them from nearly all other components save ammonia, hence the problem was to find a reagent which would discriminate between ammonia and the amines. Weber & Wilson (1918) reported that mercuric oxide would adsorb ammonia but not the amines, and Pugh & Quastel (1937) suggested this as a means of separating and estimating the amines. We attempted to devise a micro-method embodying this principle, but found it difficult to prepare mercuric oxide which would completely remove ammonia without adsorbing significant amounts of amine as well. After trying various reagents known to react with $-NH_2$ or ammonia (picryl chloride, toluene-*p*-sulphonyl chloride, Nessler's reagent) 1-chloro-2:4-dinitrobenzene (CDNB) proved suitable. This reacts with aliphatic amines to give *N*-alkylanilines (Willgerodt, 1876):

$$R.NH_2 + C_6H_3(NO_2)_2Cl = R.NH.C_6H_3(NO_2)_2 + HCl.$$

Blanksma & Schreinmachers (1933) determined the velocity constants for the reaction of CDNB with the amines, with M CDNB and 2M amine in 50% (v/v) ethanol at 25°. They found values of the order $k = 0{\cdot}055$ for *n*-butylamine, *n*-propylamine and *iso*amylamine. With ammonia the reaction was much slower (k 0·0024). CDNB should therefore be suitable for differentiating between ammonia and the amines, and we have found this to be so in practice. The chlorodinitroanilines are yellow compounds, and Duin (1953) has used them for the chromatographic separation and characterization of the amines. We have found that they are suitable for colorimetric determination. The reaction is not stoicheiometric but the colour developed is proportional to amine concentration over a wide range, and replicate assays—even when performed on different occasions—agree well.

EXPERIMENTAL

Preparation of dinitroanilines. A number of 2:4-dinitroanilines were prepared (Willgerodt, 1876) by condensing CDNB with the appropriate amine. Derivatives of several primary amines and of dimethylamine and diethylamine were prepared (Table 1). Di-*n*-propylamine and di-*n*-butylamine also react readily (see Fig. 3), but di*iso*propylamine and di*iso*butylamine and adrenaline both failed to react appreciably. Ammonia itself does not react readily, a yield of only 3% of dinitroaniline being obtained even after refluxing for 7 hr. (cf. Blanksma & Schreinmachers, 1933). The derivatives of the primary amines gave absorption spectra which are very similar to each other (Fig. 1). The two secondary amines examined gave slightly stronger absorption, but the form of the curve was similar. *N*-*n*-Butyl-2:4-dinitroaniline (Fig. 1) gave $E^{1\%}_{1\,cm.} = 725$, and $\epsilon = 17\,400$ at $\lambda_{max} = 358$ mμ. This would normally be the best wavelength for determinations but in practice it is better to use a longer wavelength. CDNB itself has an appreciable absorption at 358 mμ. and as it has to be used in large excess this makes reading difficult at the $\lambda_{max.}$. Examination of the spectra shows that the region of 450 mμ.

Table 1. *Properties of* 2:4-*dinitroanilines* [$RR'.N.C_6H_3(NO_2)_2$]

Extinction coefficients were measured at $\lambda_{max.}$.

R	R′	M.p. Found	M.p. Reference	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	ϵ
n-C_3H_7-	H	98°	97° (1)	358	827	18 700
n-C_4H_9-	H	92	90 (2)	358	725	17 400
$(CH_3)_2.CH.CH_2$-	H	80	80 (3)	358	777	18 600
$(CH_3)_2.CH.CH_2.CH_2$-	H	98	97 (2)	358	695	18 200
$C_6H_5.CH_2.CH_2$-	H	154	154 (4)	348	686	19 100
CH_3-	CH_3-	74	(5)	370	900	19 000
C_2H_5-	C_2H_5-	80	(5)	370	900	21 500

References: (1) Romberg & Jansen (1911). (2) Kam (1926). (3) Romberg (1885). (4) Jansen (1931). (5) Romberg (1881) reports m.p. 87° and 80° for the dimethyl and diethyl compounds respectively. Blanksma & Schreinmachers (1933) state that the compounds are dimorphic with m.p. 87° and 74° (dimethyl) and 80° and 69° (diethyl).

gives the best ratio between the absorption of CDNB itself and the derivatives of the primary amines. The absorption of the latter is still sufficient to permit adequate sensitivity ($E^{1\%}_{1\,cm.}$ = 150 at 450 mμ.). Ammonia, moreover, will not interfere at 450 mμ. If present, it will form appreciable amounts of 2:4-dinitroaniline; this has $E_{max.}$ at 335 mμ. (ε = 19 200) and would interfere with readings taken at 358 mμ.; but at 450 mμ. its absorption is negligible. Experiment showed that NH_3, even in large excess, does not interfere with readings taken at 450 mμ.

Reagent. 1-Chloro-2:4-dinitrobenzene (0·5 %) is dissolved in 50 % (v/v) aqueous propan-1-ol and stored in a dark bottle. It is stable for at least two months but should be discarded if excessive blank values are obtained.

Spectrophotometry. All measurements were made in a 1 cm. cell in either a Beckman or a Unicam instrument.

Technique. The sample for assay, containing 50–250 μg. of amine N in a volume not exceeding 10 ml., is placed in a suitable micro-steam-distillation apparatus, such as that described by Markham (1942). Propan-1-ol (2 ml.) is placed in the receiver and steam passed until approx. 5 ml. of distillate has been collected over a period of 2 min. The distillate is transferred to a 6 in. × $\frac{5}{8}$ in. test-tube, graduated at 10 ml., and 1 ml. of the CDNB reagent added. At the same time a standard is prepared containing 140 μg. of amine N in 5 ml. of 50 % (v/v) propan-1-ol. The amine used for the standard should preferably be an authentic sample of the amine being assayed; otherwise, *n*-butylamine is a convenient standard. The CDNB reagent (1 ml.) is added to the standard, and also to a blank consisting of 5 ml. of 50 % (v/v) propan-1-ol. Assay, standard and blank are placed in a bath containing cold water and the mouths of the tubes covered with loose glass stoppers to prevent loss of solvent. The bath is allowed to stand at room temperature for 15 min. and is then gradually raised to the boil over a period of 0·5 hr., and held at 100° for 0·5 hr. The tubes are then cooled and made up to the 10 ml. mark with 50 % (v/v) propan-1-ol. Standard and unknown are read against the blank in a spectrophotometer at 450 mμ. The blank should also be read against the solvent [50 % (v/v) propan-1-ol]; this reading ($\log_{10} I_0/I$) should not exceed 0·12 in a 1 cm. cell.

Time of heating. The technique described, involving standing at room temperature and slowly heating to 100°, avoids loss of the volatile amine before reaction has taken place. If the tubes are placed directly in a boiling-water bath results are erratic and tend to be low. The procedure described ensures uniform results. Similarly, propan-1-ol is chosen as solvent because of its miscibility with water combined with relatively high boiling point. During the final heating at 100°, however, the colour is fully developed after about 15 min., and there is no further change if heating is prolonged beyond the specified time.

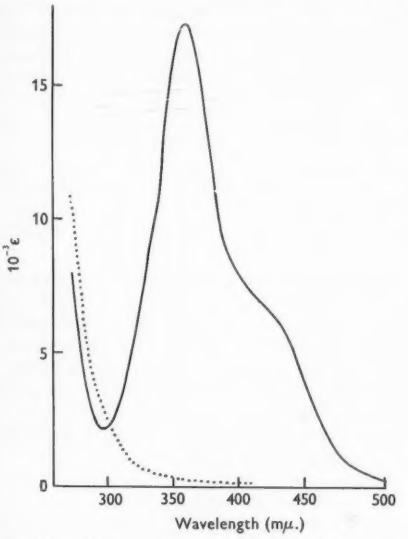

Fig. 1. Absorption spectra of 1-chloro-2:4-dinitrobenzene (···) and *N*-*n*-butyl-2:4-dinitroaniline (——) in 50 % (v/v) ethanol, measured in a 1 cm. cell in a Beckman spectrophotometer.

RESULTS

Specificity of reaction. Fig. 2 shows that the three primary aliphatic amines give approximately equal colour intensities, β-phenylethylamine about 25 % less. Fig. 3 gives the results with some secondary aliphatic amines. The method would probably be suitable for estimation of some secondary amines, though we have made no detailed investigations. Di*iso*propylamine and di*iso*butylamine gave no appreciable colour. Ammonia gave about 0·1 %, indole 2·5 %, and skatole 4 % of the readings obtained with *n*-butylamine.

Linearity of response. Figs. 2 and 3 show that absorption is proportional to the amount of amine present up to at least 300 μg. of amine N. As this

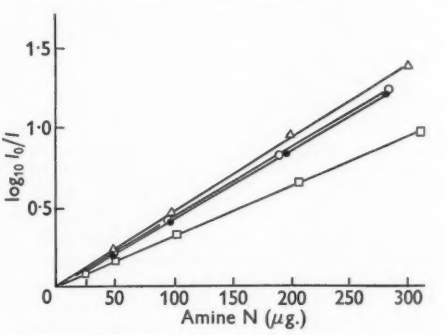

Fig. 2. Colour development with primary amines. Varying quantities of the amines were treated with CDNB in accordance with the assay procedure and the absorption was read at 450 mμ. Each point represents the mean of a pair of replicate readings. ○, *n*-Propylamine; ●, *n*-butylamine; △, *iso*amylamine; □, β-phenylethylamine.

Table 2. *Reproducibility of results of assay of* n-*butylamine*

The quantities of amine (expressed as N) in the first column were subjected to the assay procedure. Duplicates were set up and the absorptiometer readings (at 450 mμ.) are recorded in columns A1 and A2. The following day the experiment was repeated and the results are given in columns B1 and B2; a third repetition on another occasion gave the results in columns C1 and C2. The mean was calculated, and the last column gives the standard percentage error for the mean of any two readings.

Amine N (μg.)	A1	A2	B1	B2	C1	C2	Mean	S.E. (%)
24	0·115	0·110	0·114	0·092	0·097	0·100	0·105	4·1
47·5	0·220	0·206	0·210	0·203	0·210	0·200	0·212	2·1
95	0·425	0·411	0·410	0·402	0·410	0·410	0·411	2·6
190	0·830	0·825	0·800	0·810	0·810	0·825	0·815	1·8
285	1·31	1·30	1·20	1·20	1·21	1·22	1·24	5·6

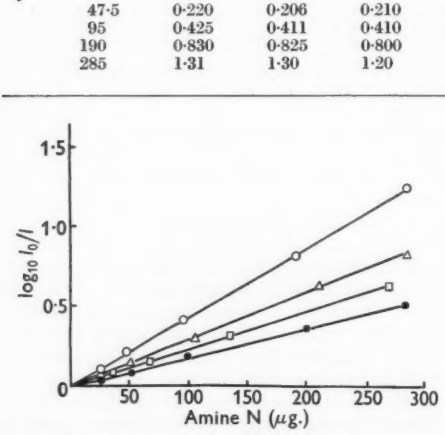

Fig. 3. Colour development with secondary amines. ○, Di-*n*-butylamine; △, di-*n*-propylamine; □, dimethylamine; ●, diethylamine.

amount gives about the maximum absorption which can be read satisfactorily in the spectrophotometer, larger amounts were not investigated.

Consistency of results. Since the reaction is not stoicheiometric it is to be expected that the colour developed might vary with slight changes in the conditions of assay. It is therefore essential that a standard should be set up with each batch of assays. The extent of fortuitous variation was investigated by setting up three pairs of replicate assays on three separate days. The results are given in Table 2. The standard deviations of each set of six results were calculated, and from this the standard error (P 0·05) for the mean of any pair of readings. This figure (expressed as a percentage of the reading) is given in the last column of Table 2. It shows that if assays are performed in duplicate and the mean is taken, an accuracy of about ± 2 % is attainable over the best part of the range.

DISCUSSION

Comparison of the colour developed in the assay with the extinction coefficients of the 2:4-dinitroanilines shows that only about one-sixth of the amine is converted into the derivative under these conditions. The colour developed is, however, almost proportional to the concentration of CDNB even when the latter is in large excess. This suggests that the reaction proceeds to an equilibrium. Attempts were made to drive the reaction nearer completion. Increasing the concentration of CDNB was not possible, since the reagent was already almost a saturated solution; use of a larger volume would dilute the amine and the overall gain would be small. Use of more CDNB would also increase the blank. A more attractive method of driving the reaction forward was to seek some method of removing the hydrochloric acid formed from the system. This could not, however, be done by addition of alkali, which gave an intense yellow colour in the blank (presumably owing to hydrolysis of the CDNB), nor by tertiary amines such as pyridine, quinoline or triethanolamine which also gave excessive blanks. Replacement of the CDNB by fluorodinitrobenzene was also tried, but it gave a highly coloured derivative with ammonia. Even if the reaction could be made to proceed to completion, however, the gain would be solely in the sensitivity of the method; its accuracy and specificity have already reached the limits normally attained in a method of this kind.

Though the method, as described, depends on the use of a spectrophotometer, a simpler instrument will give satisfactory results. We have used the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, England) with a narrow-band blue filter (EEL no. 621). The range over which a linear response is obtained is somewhat restricted, however, and will depend on the characteristics of the instrument and filter used.

SUMMARY

A method is described for the micro-determination of lower aliphatic amines in biological material. The amine is steam-distilled at alkaline pH and allowed to react with 1-chloro-2:4-dinitrobenzene. The

yellow product is estimated spectrophotometrically. Ammonia does not interfere with the determination.

We are deeply indebted to Profressor R. A. Morton, F.R.S., for his advice and encouragement, and to the Medical Research Council for a grant towards the expenses of this investigation.

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Studies on Sulphatases

14. A PRELIMINARY ACCOUNT OF THE CHONDROSULPHATASE OF *PROTEUS VULGARIS**

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The existence in putrefactive bacteria of an enzyme which liberated sulphuric acid from chondroitin sulphate was first suspected by Neuberg & Rubin (1914). Subsequently, a concentrate of this enzyme, to which the name 'chondrosulphatase' was given, was obtained from an organism resembling *Bacterium fluorescens* 'non-liquifaciens' (syn. *Pseudomonas fluorescens* 'non-liquifaciens') (Neuberg & Hofmann, 1931*a*, *b*). Other putrefactive bacteria, *Bacterium proteus* (syn. *Proteus vulgaris*) and *Bact. pyocyaneus* (syn. *Pseudomonas aeruginosa*) were also shown to possess chondrosulphatase activity (Neuberg & Hofmann, 1931*a*, *b*). The ability of micro-organisms, including those isolated from the oral flora (Pincus, 1950), to desulphate mucopolysaccharides isolated from human enamel and dentine (Pincus, 1949; Candelli & Tronieri, 1951) has suggested a relationship between bacterial chondrosulphatase and the production of dental caries. A chondrosulphatase is present in living cultures of *Penicillium spinulosum* (Pincus, 1950) and in the digestive organs of the marine mollusc *Charonia lampas* (syn. *Triton nodiferus*) (Soda & Egami, 1938), but it is not yet clear whether the enzyme is present in mammalian tissues. Thus although no activity could be detected in a number of different rat tissues by sensitive chemical or histochemical methods (Dohlman & Friedenwald, 1955), it was possible to detect appreciable amounts of $^{35}SO_4^{2-}$ in rat urine after the administration of ^{35}S-containing chondroitin sulphate (Dr C. H. Dohlman, personal communication). There is also a certain amount of somewhat confused evidence which suggests that the enzyme elastase contains more than one component, one of which may be a chondrosulphatase type of enzyme (Pepler & Brandt, 1954; Hall & Gardiner, 1955).

In the studies mentioned above and in other investigations on chondrosulphatase activity in bacteria (Beuhler, Katzman & Doisy, 1951; Reggianini, 1950*a*, *b*; Konetzka, Pelczar & Burnett, 1954) liberation of sulphuric acid from chondroitin sulphate was always accompanied by the appearance of degradation products possessing reducing activity. Although the mucopolysaccharase (chondroitinase) system responsible for the appearance of reducing activity has in no case been completely separated from the associated chondrosulphatase, in the mollusc preparations a partial separation of the two enzymes has been achieved and there seems to be no doubt as to their separate identities (Soda & Egami, 1938).

In spite of the possible value of a chondrosulphatase as a tool in establishing the structure of chondroitin and related sulphates little is known of the properties of the enzyme. The present report presents the results of a preliminary study of the chondrosulphatase of a strain of *Proteus vulgaris*.

* Part 13: Dodgson, Spencer & Williams, (1956).

MATERIALS AND METHODS

Preparation of potassium chondroitin sulphate. Recently three different types of chondroitin sulphate have been recognized (Meyer & Rapport, 1951; Orr, 1954), and it seems probable that most preparations obtained by simple extraction procedures are unlikely to be homogeneous. However, for these preliminary studies complete homogeneity of substrate is not essential and the product prepared by a modification of the method of Einbinder & Schubert (1950) has been used.

The rings of cartilage from fresh bovine trachea were immersed for 10 min. in acetone, when the hardened non-cartilaginous material could readily be removed. The cartilage was washed, coarsely minced, and 150 g. of the mince was macerated in 200 ml. of water containing 0·25 g. of crude trypsin (British Drug Houses Ltd.) and 0·1 g. of thymol, the whole being adjusted to pH 7·8. After incubating at 37° for 72 hr., 1 l. of a 30% (w/v) solution of KCl was added and the mixture shaken mechanically for 72 hr. at room temperature. The cloudy extract was decanted and stored at 0° while the residue was shaken with a further 1 l. of KCl solution for 72 hr. The two extracts were combined and dialysed for 40 hr. against tap water in order to reduce the salt concentration of the solution and thereby increase the efficiency of the subsequent deproteinization procedure (Strandberg, 1951). Acetic acid (20 ml.) and 20 g. of potassium acetate were added and the solution was stirred for 30 min. with 20 g. of kaolin. The bulk of the kaolin was removed by centrifuging, the solution clarified by filtering through Hyflo Super-Cel (Johns Manville Inc.) and the filtrate dialysed for 24 hr. against tap water. The treatment with acetic acid, potassium acetate and kaolin was repeated, the solution clarified as before and then concentrated to about 300 ml. *in vacuo* at 40°. The solution was poured, with stirring, into 1 l. of ethanol and the whole allowed to stand overnight at 0°. The white flocculent precipitate of potassium chondroitin sulphate was separated, washed well with ethanol and ether and dried *in vacuo*. The product was dissolved in water to give a 3% (w/v) solution, acidified with acetic acid (2 ml./100 ml.) and stirred with kaolin (4 g./100 ml.) for 30 min. The solution was clarified as described above, stirred with a further quantity of kaolin (4 g./100 ml.), clarified again and the filtrate dialysed against several changes (100 vol. in all) of distilled water. Potassium acetate (10 g.) was added to each 100 ml. of diffusate and the potassium chondroitin sulphate precipitated by the addition, with stirring, of 3 vol. of ethanol. The precipitate was separated, washed with ethanol followed by ether, and dried *in vacuo*. Yield, 5–7 g. Analysis (average of three preparations): N, 2·23% (determined by the method of Markham, 1942); SO_4^{2-} ions, 14·19% (determined by the gravimetric procedure of Lugg, 1938, after hydrolysis by refluxing with 4N-HCl for 20 hr.); hexosamine, 26·0% (determined according to Belcher, Nutten & Sambrook, 1954); N:S ratio, 1·08.

Viscosity measurements. The viscosity of solutions of potassium chondroitin sulphate were determined with an Ostwald viscometer having a flow time of 50 sec. for 2 ml. of water. The temperature was maintained at 37° (±0·05°) and the time of flow was measured to the nearest 0·1 sec.

Choice of buffer concentration for enzyme experiments. Mathews (1953) has shown that the viscosity behaviour of solutions of chondroitin sulphate is typical of that of linear polyanions and postulated that the shape of the chondroitin sulphate molecule varies from that of a loose flexible coil at low cation concentration to that of a tightly coiled sphere or near-sphere at high cation concentration. At pH 7·0 the viscosity of solutions of potassium chondroitin sulphate (0·095 or 0·64%, w/v) in sodium acetate–acetic acid solution was independent of the concentration of acetate over the range 0·2–0·5M. Between these limits it can therefore be assumed that there is no gross change in the shape of the chondroitin sulphate molecule and consequently in the spatial distribution of the ester sulphate groupings. A concentration of acetate of 0·2M was chosen for use in the enzyme determinations in order that cations which may be present in the enzyme preparations should not influence the molecular shape. In many of the enzyme experiments acetate solutions were used at pH greater than 7·0. At pH values greater than 6·0 acetate mixtures have little buffering power and in these cases the pH of the incubation mixture was checked at the beginning and the end of the incubation period. No detectable changes in pH were observed.

Measurement of chondrosulphatase activity. Chondrosulphatase activity was measured by estimating the enzymically liberated sulphate by the benzidine micromethod of Dodgson & Spencer (1953). The concentration of the various enzyme preparations was adjusted so that between 20 and 40 μg. of SO_4^{2-} ions was liberated in the incubation mixture. In the original account it was reported that the method gave anomalous results when concentrations of potassium chondroitin sulphate greater than 0·5% (w/v) were present in the incubation mixture. It is now clear that an impure preparation of chondroitin sulphate was used in these early studies, and more recent work has shown that with freshly prepared potassium chondroitin sulphate, quantitative recoveries of added SO_4^{2-} ions could only be obtained when the concentration of chondroitin sulphate in the incubation mixture was less than 0·17% (w/v). When it was necessary to use higher concentrations the original method had to be modified, as described for the assay of glycosulphatase by Dodgson & Spencer (1954). In such cases instead of incubating 0·6 ml. of acetate containing potassium chondroitin sulphate solution with 0·6 ml. of acetate containing enzyme solution, smaller volumes of substrate (0·1 ml. or 0·2 ml.) were incubated with an equal volume of a proportionately more concentrated enzyme solution and the modified procedure then followed (see Dodgson & Spencer, 1954).

Measurement of mucopolysaccharase activity. A variety of methods, both physical and chemical, have been developed for the assay of mucopolysaccharase systems (see Meyer & Rapport, 1952). In the present case the estimation of liberated reducing substances was considered to be the most convenient. The method used was as follows: the enzyme solution (0·6 ml. in 0·2M acetate solution, pH 7·0), contained in a 15 ml. centrifuge tube, was pre-incubated for 3 min. at 37° before the addition of 0·6 ml. of the substrate in 0·2M acetate solution, previously warmed to 37°. After incubation at 37° for the required period, the reaction was stopped by the addition of 2·4 ml. of 0·3N-NaOH, followed immediately by 2·4 ml. of a 5% (w/v) aqueous solution of $ZnSO_4,7H_2O$, these reagents having previously been standardized against each other as recommended by Somogyi (1945). Suitable control determinations were made in which enzyme and substrate were incubated separately and mixed only immediately before the addition of the

NaOH. The precipitate was removed by centrifuging at 2500 *g* for 20 min., and a portion (2·5 ml.) of the clear supernatant was mixed with 2·5 ml. of Somogyi (1952) copper carbonate reagent in tubes chosen for uniformity of diameter and wall thickness and graduated to 7 ml. The mixture was heated for 10 min. in a boiling-water bath, the mouth of the tube being closed with a glass ball to prevent excessive loss by evaporation. The tube was cooled in running water and 2 ml. of arsenomolybdate reagent (British Drug Houses Ltd., see Nelson, 1944) added. After standing for at least 12 hr. at 2°, the volume was adjusted to 7 ml. with water and the colour densities of both test and control determinations were measured against a reagent blank in 0·5 cm. cells at 750 mμ., with the Unicam SP. 600 spectrophotometer. Anhydrous D-glucose (Thomas Kerfoot and Co. Ltd.) standards, in the range 10–150 μg., were treated in the manner described above and a calibration curve was constructed. The results of the enzyme assays were then expressed as μg. of reducing substance, with glucose as the reference standard. Added glucose could be quantitatively recovered from enzyme solutions in acetate over a wide range of substrate concentrations.

RESULTS

Estimation of the chondrosulphatase activity of growing cultures

A number of micro-organisms obtained from the National Collection of Type Cultures were tested for chondrosulphatase activity in the following way. Sterile nutrient broth (1·0% of Oxoid peptone, 0·3% of Oxoid Lab Lemco and 0·1% of NaCl, all w/v in water) was adjusted to pH 7·0 with N-NaOH or N-HCl, inoculated with the test organism and incubated at 25° for 48 hr. A Roux flask was sterilized by dry heat and sterile nutrient agar added to form a thin layer on one side. The broth culture (5 ml.) was added to the flask, spread evenly over the agar surface and incubated at 25° for 24 hr. The cells were washed from the agar with four 5 ml. portions of sterile water, and the suspension of cells so obtained was centrifuged at 4500 *g* and 0° for 45 min. The precipitated cells were washed twice with sterile water (5 ml.) and then suspended, in a sterile glass homogenizer, in 10 ml. of sterile 0·2 M acetate solution, pH 7·0. To a sterile 15 ml. centrifuge tube containing 0·2 ml. of a 0·75% (w/v) solution of potassium chondroitin sulphate in 0·2 M acetate solution, pH 7·0, which had been previously sterilized by Seitz filtration, was added 0·2 ml. of the cell suspension. The tube was incubated for 12 hr. at 37° and the enzymically liberated sulphate estimated by the benzidine micro-method modified for this high substrate concentration as described above. Determinations were carried out in duplicate with suitable controls.

For the determination of dry weight 0·2 ml. of the cell suspension and 0·2 ml. of the substrate solution were incubated as above in a weighed sterile tube. Substrate was included in case appreciable bacterial growth occurred under these conditions during the incubation period. The cells were collected by centrifuging, washed several times with water and the tubes dried overnight at 105° before weighing. Results are recorded in Table 1.

Table 1. *Chondrosulphatase activity of certain bacteria*

National Collection of Type Cultures no.	Organism	Sulphate liberated in 12 hr. (μg./mg. dry wt. of cells)
4175	*Proteus vulgaris* I	19·4
4636	*Proteus vulgaris* I	102·5
8311	*Proteus vulgaris* II	29·0
8313	*Proteus vulgaris* II	51·2
5940	*Pseudomonas fluorescens*	0
6751	*Pseudomonas fluorescens*	0
912	*Pseudomonas ovalis*	0
7914	*Pseudomonas ovalis*	0
5731*	*Alcaligenes metalcaligenes*	0

* National Collection of Industrial Bacteria no.

Purification and properties of the chondrosulphatase of Proteus vulgaris (*N.C.T.C.* 4636)

Table 1 shows that *P. vulgaris* 4636 was particularly rich in chondrosulphatase activity, and this organism was selected as a suitable source of the enzyme. The organism was cultured as follows: large flasks, each containing 16 l. of nutrient broth (see previous section), were sterilized. To each flask was added 20 ml. of a 24 hr. broth culture of the organism and the flasks were incubated at 25°, aeration and stirring being provided by a sterile air stream. Maximum growth was obtained after 5 days, when the cells were harvested with the aid of a Sharples centrifuge, suspended in water and again separated by centrifuging. The cells were macerated in acetone at 0°, filtered at the pump, washed well with acetone at 0° and dried *in vacuo*. Yield, 4–5 g./16 l. of broth (preparation *A*). During later stages of the work acetone-dried cells from large-scale fermentations (320 l.) of the organism were prepared in analogous fashion at the M.R.C. Antibiotic Research Station, Clevedon.

Properties of preparation A. Maximum chondrosulphatase activity was obtained at pH 7·0 (Fig. 1) when measured over a period of 1 hr. at 37° with a final substrate concentration of 0·045% (w/v) in the presence of 0·2 M acetate solution. Although the pH curve did not vary when the concentration of acetate was increased to 0·5 M the enzyme activity was a little lower. At pH 7·0 maximum enzyme activity was obtained at a substrate concentration of 0·045% (w/v) (Fig. 2). Under these optimum conditions the chondrosulphatase activity of a number of different samples of preparation *A* was approximately 2 μg. of sulphate liberated/hr./mg.

The optimum conditions did not vary from preparation to preparation.

The optimum pH for mucopolysaccharase activity with a substrate concentration of 0·045% was greater than 7·5. However, no attempt was made to establish the actual optimum pH and all determinations of mucopolysaccharase activity were made with the optimum conditions for chondrosulphatase activity. Under these conditions preparation *A* liberated 5·8 μg. of reducing substance/hr./mg.

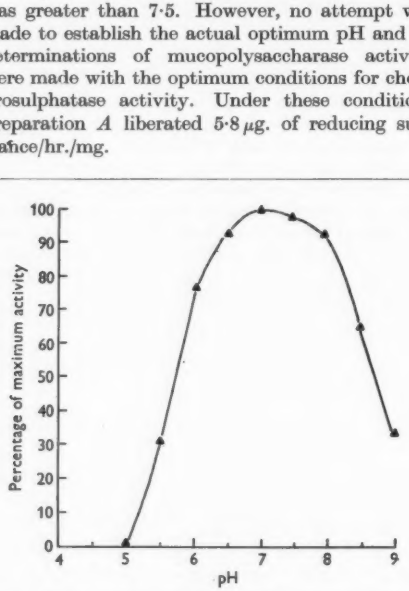

Fig. 1. pH–Activity curve of the chondrosulphatase of *P. vulgaris* (preparation *A*) acting on 0·045% (w/v) potassium chondroitin sulphate in 0·2M acetate solution over 1 hr. at 37°.

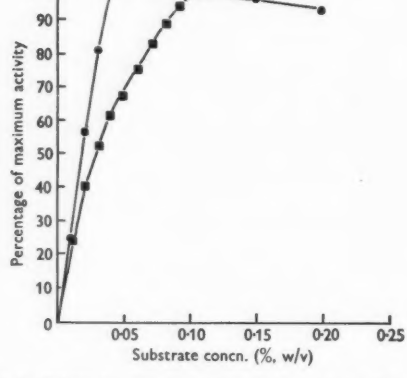

Fig. 2. Substrate concentration–activity curves for the chondrosulphatase of *P. vulgaris* (preparation *A*, ●; *B*, ■) acting on potassium chondroitin sulphate in 0·2M acetate, pH 7·0. Incubation was for 1 hr. at 37°.

Solubilization of the chondrosulphatase. Homogenization of water suspensions of the acetone-dried cells in a glass homogenizer, followed by standing at 2° for periods of 2–8 hr., did not liberate the chondrosulphatase into solution. Grinding in a bacterial mill (Booth & Green, 1938) was equally unsuccessful. Alternate freezing and thawing followed by standing at 2° for 5 hr. released 15% of the enzyme activity into solution. Incubation in water at 25° or 37° did not solubilize the enzyme but incubation at 37° in 0·2M acetate solutions at pH 7·0–8·0 liberated considerable amounts of the enzyme into solution. The behaviour of the mucopolysaccharase activity during these various treatments closely paralleled that of the sulphatase.

Preparation *A* was homogenized in 0·2M acetate solution, pH 7·0 (1 g. of *A*/20 ml. of solution), the suspension was re-adjusted to pH 7·0 and the whole incubated for 2 hr. at 37°. The cellular debris was removed by centrifuging at 18 000 ***g*** and 2° for 20 min., and the supernatant solution was dialysed against several changes of distilled water over a period of 16 hr. in the refrigerator. The diffusate was clarified by centrifuging at 18 000 ***g*** and 2° to give a cell-free extract which was used as the starting material for further purifications. The white powder obtained by freeze-drying this extract (preparation *B*, yield about 0·1 g./g. of *A*) was completely soluble in water.

Properties of preparation B. At a substrate concentration of 0·045% (w/v) preparation *B* gave a pH curve which was identical with that obtained with preparation *A* (Fig. 1). However, the substrate concentration–activity curve at pH 7·0 was quite different from that obtained for preparation *A* (see Fig. 2) and maximum enzyme activity was now obtained at a substrate concentration of 0·125% (w/v). With this concentration of substrate the optimum pH remained unchanged at 7·0. Several different preparations of *B* gave the same optimum pH and substrate concentration and under these conditions possessed an average activity of 19 μg. of sulphate liberated/hr./mg. The mucopolysaccharase activity, when measured under the same conditions, was 32 μg. of reducing substance/hr./mg.

pH *Inactivation and precipitation.* The extent to which chondrosulphatase and mucopolysaccharase were inactivated over 1 hr. at 37° was identical, within the limits of experimental error, over the whole of the pH range tested, both enzymes being completely inactivated at pH lower than 5 or greater than 11. However, at 0° the enzymes were less sensitive to pH inactivation and at pH below 5·8 material containing both chondrosulphatase and mucopolysaccharase was precipitated from a 0·5% (w/v) water solution of preparation *B*. The precipitation of both enzyme activities was closely parallel throughout the pH range tested, maximum pre-

cipitation occurring at pH 4·0. At this pH the precipitated material contained 75 % of the total sulphatase activity and 70 % of the total mucopolysaccharase activity. The extent to which the enzymes were precipitated at pH 4·0 was decreased in the presence of 0·2 M acetate solution. The material precipitated at pH 4·0 contained appreciable amounts of nucleic acid as shown by the ultraviolet-light absorption spectrum. After removal of this nucleic acid from solutions of the precipitated material by the addition of protamine sulphate, the enzyme activities could no longer be precipitated by adjusting the pH to 4·0. This suggested that the initial precipitation of the enzymes at this pH might be dependent on the presence of nucleic acid and that more complete precipitation might be achieved by increasing the concentration of nucleic acid. Addition of yeast nucleic acid (L. Light and Co. Ltd.) before adjusting to pH 4·0 increased the precipitation of the sulphatase to 93 % and the mucopolysaccharase to 87 %. On the basis of these results preparation *B* was further purified in the following manner.

The dialysed cell-free extract (preparation *B* before freeze-drying) was cooled to 2° and the sodium salt of yeast nucleic acid [0·2 ml. of a 5 % (w/v) aqueous solution at pH 7·4/20 ml. of extract] was added. The pH of the mixture was adjusted to 4·0 with acetic acid and the whole allowed to stand for 30 min. at 2°. The precipitated material was removed by centrifuging at 4 500 ***g*** and 2° for 30 min., suspended in one-fifth of the original volume of water, and dissolved by adjusting the pH to 7·4 with N-NaOH. The solution was adjusted to pH 6·7 with N-HCl and an aqueous solution of protamine sulphate (1 %, w/v), adjusted to the same pH, was added until a portion of the clear supernatant obtained by centrifuging at 18 000 ***g*** for 5 min. at 2° no longer gave a precipitate on adding further protamine sulphate solution. The pH of the nucleic acid-free supernatant was adjusted to 8·0 with N-NaOH, and the whole cooled to 2°. Any excess of protamine sulphate which was precipitated was removed by centrifuging at 18 000 ***g*** at 2° for 20 min., and the supernatant was dialysed against 20 vol. of distilled water at 2° for 12 hr. A small amount of insoluble material separated out during dialysis and was removed by centrifuging. The diffusate was freeze-dried to give a white powder (preparation *C*) which was completely soluble in water. The average yield was 100 mg./10 g. of preparation *A*.

Properties of preparation C. The optimum conditions for the chondrosulphatase were identical with those for preparation *B*, and under these conditions the activity corresponded to a liberation of 65 μg. of sulphate/hr./mg. The mucopolysaccharase activity corresponded to the release of 107 μg. of reducing substance/hr./mg. The ability of preparation *C* to liberate sulphate from the sodium or potassium salts of a number of synthetic or naturally occurring sulphate esters was measured at 37° over a period of 24 hr. No sulphatase activity towards any of the compounds tested (Table 2) was observed.

Table 2. *Sulphate esters not attacked by the chondrosulphatase of* Proteus vulgaris **4636**

Preparation *C* (0·6 mg. in 0·6 ml. of 0·2 M acetate, pH 7·0) was incubated with 0·6 ml. of a solution of the sulphate ester in the same solution. Except where otherwise stated the concentration of the sulphate ester in the incubation mixture was 0·1 % (w/v), and under such conditions added sulphate could be quantitatively recovered. In no case was enzymic liberation of sulphate observed, even after incubation for 24 hr. at 37°.

Polysaccharide sulphates:
Heparin
Carragheenin
Agar
Fucoidin
Chondrus ocelatus mucilage (Mori, 1953)
Other carbohydrate sulphates:
Glucose 6-sulphate (0·01 M)
Uridine diphosphate acetylgalactosamine sulphate (0·8 mM)
Acetylgalactosamine sulphate (0·8 mM)
Aryl sulphates:
2-Hydroxy-5-nitrophenyl sulphate (0·02 M)
p-Nitrophenyl sulphate (0·0025 M)
p-Acetylphenyl sulphate (0·007 M)
Tyrosine *O*-sulphate (0·02 M)
Steroid sulphate:
Dehydro*isoepi*androsterone sulphate (0·2 mM)
Mustard-oil glycoside sulphate:
Sinigrin (potassium myronate)
Alkyl sulphates:
Ethyl sulphate
Choline sulphate

Time course of the enzymic degradation of chondroitin sulphate

During the concentration procedures no appreciable separation of chondrosulphatase and mucopolysaccharase activities was achieved and it was not possible therefore to gauge the extent to which the activities of the two enzymes were interdependent. With this point in mind the time–activity curves of the enzymes were examined (Figs. 3, 4). The curves for preparation *A* (Fig. 3, curves 1, 2 and 3) show that, whereas liberation of material possessing reducing activity begins immediately, there is a distinct lag in the appearance of inorganic sulphate. This lag is prolonged by increasing the concentration of chondroitin sulphate in the incubation mixture. These findings suggested that sulphatase activity was dependent on preliminary mucopolysaccharase attack. However, when the

experiments were repeated with preparation *B* (Fig. 4), no such time-lag was observed. The essential difference between preparations *A* and *B* is that, in the latter, sulphatase and mucopolysaccharase have been extracted from the acetone-dried cells by incubation with acetate solution and subsequently separated from the cell debris. Preliminary incubation of preparation *A* for 2 hr. with 0·2 M acetate solution, pH 7·0, before addition of substrate, eliminated the lag phase completely (Fig. 3, curve 4) irrespective of the concentration of chondroitin sulphate subsequently used in the assay procedure. Removal of the lag phase could not be achieved by carrying out a similar incubation in water, and it is known that this treatment does not extract the sulphatase and mucopolysaccharase from the acetone-dried cells.

It has also been shown that during the extraction of the two enzyme systems from the acetone-dried cells by pre-incubation with acetate, polysaccharide material, which is present in preparation *A*, is degraded to give material possessing reducing activity. There is no detectable increase in reducing activity when the cells are incubated in water alone.

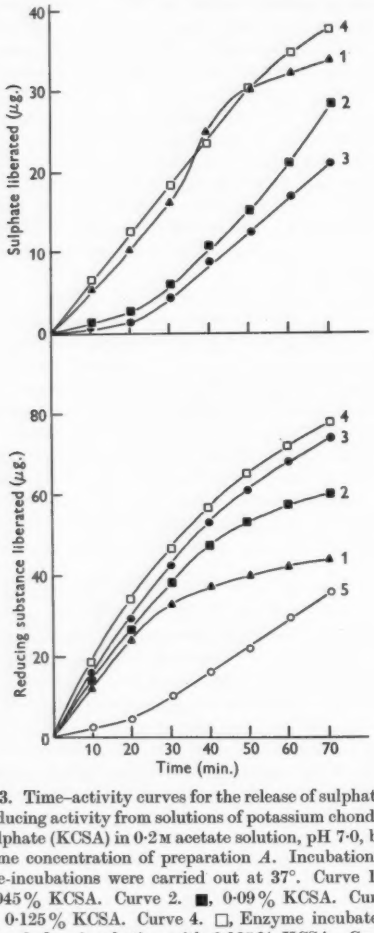

Fig. 3. Time–activity curves for the release of sulphate and reducing activity from solutions of potassium chondroitin sulphate (KCSA) in 0·2 M acetate solution, pH 7·0, by the same concentration of preparation *A*. Incubations and pre-incubations were carried out at 37°. Curve 1. ▲, 0·045% KCSA. Curve 2. ■, 0·09% KCSA. Curve 3. ●, 0·125% KCSA. Curve 4. □, Enzyme incubated for 2 hr. before incubation with 0·125% KCSA. Curve 5. ○, Release of reducing substance from the enzyme preparation incubated in the absence of KCSA.

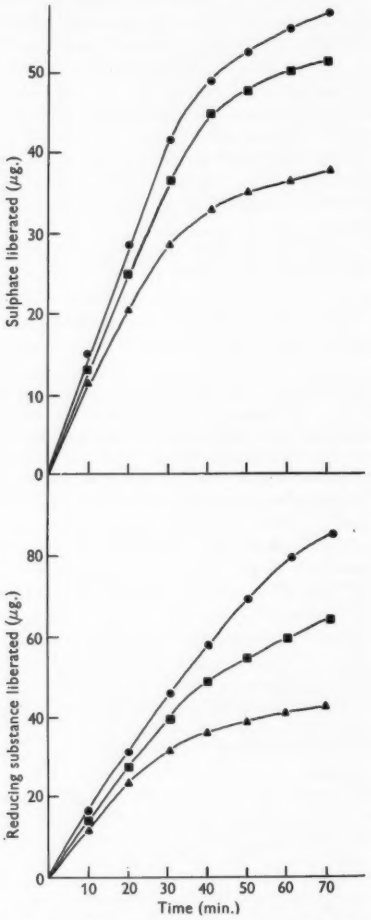

Fig. 4. Time–activity curves for the release of sulphate and reducing activity from solutions of potassium chondroitin sulphate (▲, 0·045%; ■, 0·09%; ●, 0·125%) in 0·2 M acetate solution, pH 7·0, by the same concentration of preparation *B*. Incubation was carried out at 37°.

The presence in *P. vulgaris* of polysaccharide material which can be degraded by testicular hyaluronidase has already been noted by Warren & Gray (1954). Fig. 3, curve 5, shows part of the time–activity curve for the appearance of reducing activity when preparation *A* is incubated in 0·2 M acetate solution in the absence of potassium chondroitin sulphate. Release of reducing activity is complete in 2 hr. This time course is substantially the same as that obtained when testicular hyaluronidase is also present in the incubation mixture. The degradation of endogenous polysaccharide during the incubation of preparation *A* emphasizes the need for strict control determinations when assaying the mucopolysaccharase activity of such preparations.

To summarize: during the initial stages of the incubation of preparation *A* with chondroitin sulphate in the presence of acetate solution, mucopolysaccharase activity proceeds normally but sulphatase activity progressively increases, as some change, dependent on the presence of acetate, occurs. It is possible that salt solutions other than acetate might be capable of inducing this change but this has not been tested. The rate at which the change proceeds is progressively delayed by increasing the concentration of chondroitin sulphate. The nature of the change is not clear, but increasing 'solubilization' of the enzyme and degradation of the polysaccharide material present in the acetone-dried cells of the bacterium may be involved. In preparation *B* the preliminary change has already occurred during the preparative procedure, and sulphatase activity proceeds normally. The time–activity curves obtained with *B* gave no indication that sulphatase activity is dependent on preliminary degradation of the substrate by the mucopolysaccharase system.

The anomalous time–activity curves given by preparation *A* help to explain why the apparent optimum substrate concentration obtained for this preparation differs from that obtained for *B* (Fig. 2). With *A*, increasing substrate concentration lengthens the period of lag before the appearance of full sulphatase activity, and consequently, when measured over a period of 1 hr., there is an apparently greater activity at a substrate concentration of 0·045 % than at 0·125 %. When measured over periods longer than 2 hr. greatest activity is obtained with a substrate concentration of 0·125 %, the optimum substrate concentration for preparations *B* and *C*. The anomalous time curves also explain why the substrate concentration–activity curve for preparation *A* when measured over 1 hr. shows inhibition by excess of substrate (Fig. 2). Inhibition by excess of substrate has been noted for the chondrosulphatase of viable cultures of *P. vulgaris* (Konetzka *et al.* 1954).

The rate of liberation of sulphate from chondroitin sulphate by enzyme preparations *A*, *B* and *C* decreased markedly with time and it was of interest to see whether total liberation of sulphate could be achieved or whether smaller sulphated fragments, resistant to sulphatase attack, would remain. With 2·4 mg. of preparation *B* in 1·2 ml. of acetate solution, pH 7·0, the ester sulphate present in 200 μg. of potassium chondroitin sulphate was completely liberated after incubation at 37° for 30 hr. When the amount of chondroitin sulphate present was increased to 500 μg., only 83 % of the ester sulphate was hydrolysed by the same amount of enzyme during 30 hr.

DISCUSSION

The preliminary studies on the specificity of the chondrosulphatase show it to be devoid of activity towards the substrates of aryl-, glyco-, steroid- and myro-sulphatases (cf. Buehler *et al.* 1951). Early preparations of bacterial chondrosulphatase were reported to liberate sulphate from glucose 6-sulphate and myrosin (Neuberg & Hofmann, 1931*a*), but it now seems likely that these observations were due to the presence in the preparations of other sulphatases. The fact that chondrosulphatase failed to hydrolyse the polysaccharide sulphates listed in Table 2 is perhaps not surprising, since none of these compounds contain the sulphated acetylgalactosamine residues present in chondroitin sulphate. However, in view of the recent suggestion that the uridine diphosphate acetylgalactosamine sulphate isolated from hens' oviducts might be an intermediate in the synthesis of chondroitin sulphate (Strominger, 1955), it is particularly interesting that the *Proteus* chondrosulphatase failed to liberate sulphate from this compound or from acetylgalactosamine sulphate derived from it.

No significant separation of the chondrosulphatase and mucopolysaccharase activities was achieved during the purification procedure described. The extent to which the two activities are interdependent is still therefore not clear and further attempts are now being made to separate the chondrosulphatase from the mucopolysaccharase before investigating the mechanism and specificity of the sulphatase action in greater detail.

SUMMARY

1. A number of bacteria were examined for the presence of chondrosulphatase activity. *Proteus vulgaris*, N.C.T.C. no. 4636, contained appreciable amounts of the enzyme and was selected for further study.

2. Concentrates of the chondrosulphatase have been prepared and a preliminary study of its properties has been made. Such concentrates possess

mucopolysaccharase activity which it has not so far been possible to remove.

3. The chondrosulphatase did not attack a number of other carbohydrate sulphate esters or esters which are known to be substrates for other types of sulphatases.

We are grateful for the loan of apparatus from the Royal Society, the Medical Research Council and I.C.I. Ltd. One of us (A. G. L.) is indebted to the M.R.C. for a studentship. We wish to thank the Director and Staff of the M.R.C. Antibiotic Research Station, Clevedon, for the large-scale fermentation of *Proteus vulgaris*. Gifts of chemicals are acknowledged from Drs E. T. Deward, A. B. Roy and J. L. Strominger and Professors W. T. J. Morgan and T. Mori.

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Formation of Ammonia from Glutamine by Rat-brain Particles

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Weil-Malherbe (1953) showed that in washed or dialysed rat-brain homogenates the rate of production of ammonia from glutamine was accelerated in the presence of inosine triphosphate (ITP) or of both inosinic acid and creatine phosphate. He suggested that the amide group of glutamine was transferred to ITP or another inosine phosphate to give the corresponding adenine compound, possibly at the expense of high-energy phosphate, followed by its deamination.

Reinvestigation of the problem with an enzyme preparation from rat brain apparently confirmed Weil-Malherbe's findings. However, further experiments failed to support his interpretation of the results.

It was noted that phosphate and sulphate stimulated production of ammonia under the conditions of the test (cf. Greenstein & Leuthardt, 1948). The apparent stimulation of production of ammonia from glutamine by inosine phosphates can be satisfactorily explained by the presence of phosphate and sulphate. These would be present in Weil-Malherbe's preparations. The effect of various ions on the reaction glutamine → glutamic acid + NH_3 was measured.

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MATERIALS AND METHODS

Barium creatine phosphate was prepared by the method of Ennor & Stocken (1948), and the barium salt of inosinic acid (IMP) according to Ostern (1932) (see Lehninger, 1951). The barium salt of ITP was prepared according to Kleinzeller (1942). The preparation of the sodium salts from the barium salts was achieved by decomposition with sodium sulphate. Barium creatine phosphate was dissolved in water, and barium inosine mono- and tri-phosphates were dissolved in chilled N-HCl; the barium was precipitated by the addition of a slight excess of 0·5 M-Na_2SO_4, the precipitate centrifuged off and the supernatant neutralized with NaOH (Eggleston

& Hems, 1952). Adenosine 5′-monophosphate (AMP), as the free acid, was a commercial sample (Roche Products Ltd.). Glutamine, prepared from mangolds (Hughes, 1950), was given by Dr D. E. Hughes. All solutions were brought to pH 7·4 before use.

^{32}P-Labelled IMP (Eggleston, 1954) was supplied by Mr L. V. Eggleston. The material was present on a square of chromatogram paper and gave about 30000 counts/min. The ^{32}P-labelled IMP was eluted from the paper for 10 min. by the reaction mixture in the vessel before the experiment, and the paper removed before addition of the enzyme.

Determination of ammonia. The microdiffusion method of Conway (1947) was used, the transfer time being 1 hr. at room temperature. When not in use the units were stored in 0·01 N-HCl. After diffusion of the NH_3 into the acid in the centre well, the contents were transferred to a glass-stoppered test tube, the well being washed out three times with 0·5–0·6 ml. of NH_3-free water, distilled in the apparatus described by Parnas & Heller (1924). The units were placed in a manipulator box (J. W. Towers Ltd., Widnes, Lancs.) containing an open dish of concentrated H_2SO_4, to remove traces of NH_3 from the atmosphere, during the transfer of the contents of the centre well to the test tubes. This precaution was necessary because atmospheric NH_3 caused inconsistencies. The volume was made up to 3 ml. after the addition of 0·03 ml. of 0·0003 M-$MnSO_4$. The NH_3 was estimated colorimetrically by the method of Russell (1945); 1 ml. of the alkaline phenoxide reagent and 1 ml. of the hypochlorite solution were added, and the colour was developed by standing the tubes in a boiling-water bath for 5 min. and then rapidly cooling them. The optical density at 625 mμ. was determined with a Unicam spectrophotometer against water.

Determination of phosphate compounds. Inorganic phosphate, ITP, IMP and AMP were separated and estimated by the method of Krebs & Hems (1953). The two solvents used were *iso*propyl ether–90% (w/v) formic acid (60:40, v/v) (Hanes & Isherwood, 1949) and *iso*butyric acid–NH_3 (*iso*butyric acid–N-NH_3 soln.–0·1 M disodium ethylenediaminetetraacetate; 100:60:1·6, by vol.).

Creatine phosphate (CP) was separated from inorganic phosphate by propan-1-ol–NH_3 [propan-1-ol–NH_3 soln. (sp.gr. 0·880)–0·1 M disodium ethylenediaminetetraacetate–water; 240:120:0·8:60, by vol.] (Oliver, personal communication). The solutions containing the CP were made alkaline after the precipitated protein had been removed by centrifuging and before 'spotting' on the paper.

Separation of glutamine and glutamic acid. This was achieved by the *iso*butyric acid–NH_3 solvent after 16–18 hr. on a descending chromatogram at room temperature. The compounds were located by spraying with 0·4% ninhydrin in *n*-butanol saturated with water, and heating.

Enzyme preparation. The brain of a freshly killed rat was removed and cooled in crushed ice. It was then weighed and homogenized with 5 ml. of cold water in a glass homogenizer of the Potter–Elvehjem type. To the homogenate was added sufficient water to make a concentration of 1 g. wet wt. of tissue to 10 ml. of water.

The mixture was spun in a Lusteroid centrifuge tube at 600 *g* for 3 min. in the high-speed head of an International refrigerated centrifuge. The supernatant was then spun at 8500 *g* for 15 min., the temperature being kept at 0°. The clear supernatant fluid was discarded and the particulate material resuspended in the original volume of water and spun again at 8500 *g* for 15 min. The process was repeated and the resulting particles were suspended in water to serve as enzyme preparation. Dry weights of particle preparations were obtained at 105° after 4 hr. (5·2–5·8 mg./ml.).

Samples were incubated at 30°, in 20 ml. conical flasks fitted with rubber bungs and glass tubes for gassing. Each 4 ml. of reaction mixture contained 1·3 ml. of enzyme suspension and 0·4 ml. of 0·35 M-$NaHCO_3$ buffer, pH 7·4. The final concentration of IMP, AMP and CP was 0·004 M, of glutamine, 0·008 M. The gas space contained N_2+CO_2 (95:5).

Samples (1 ml.) were removed from the vessels at the desired times and deproteinized by adding to conical centrifuge tubes containing 0·1 ml. of 30% (w/v) trichloroacetic acid. The solutions were centrifuged to remove protein and the clear supernatant was stored at −14°.

The initial concentration of NH_3 in all reaction mixtures, 1–2 μg./ml., introduced by addition of the enzyme suspension and solutions, was subtracted from the values obtained after incubation.

RESULTS

Production of ammonia from glutamine in the presence of ITP or IMP and CP together

ITP or IMP and CP together stimulated formation of NH_3 on incubation with glutamine and the enzyme preparation (Table 1) (cf. Weil-Malherbe, 1953). No production of NH_3 occurred in the absence of glutamine, indicating that this substance is the primary source of the NH_3. The rates of hydrolysis of ITP, IMP and CP were measured and also the rates of production of NH_3 from these incubated mixtures. The results are shown in Figs. 1 and 2. Fig. 1 shows that the reaction rate decreased considerably when only a small proportion of the glutamine had been broken down.

Paper chromatography of samples of each reaction mixture showed that there was no perceptible formation of AMP or adenosine during the incubation. In those reaction mixtures where IMP was initially present, inorganic phosphate increased and a quantitatively corresponding amount of IMP

Table 1. *Production of ammonia from glutamine by washed brain particles*

Volume of reaction mixture was 4 ml., including 1·3 ml. of enzyme suspension. ITP, IMP and CP, 0·004 M; glutamine, 0·008 M; $NaHCO_3$, 0·035 M; pH 7·4. Temperature of incubation, 30°.

	NH_3 produced in 60 min. (μg./ml.)
ITP + glutamine	28·2
IMP + CP + glutamine	20·1
IMP + glutamine	13·8
CP + glutamine	10·6
Glutamine	2·9
IMP + CP	0·0
IMP	0·0
CP	0·0
Nil	0·0

disappeared. In chromatograms from the same incubation mixture a spot appeared which absorbed the 257 mμ. Hg line and ran a little behind the AMP area in the *iso*butyric acid–NH_3 solvent. This spot on elution gave an ultraviolet absorption curve with a peak at 248 mμ., similar to that of hypoxanthine or inosine. The material ran in the same position as an inosine marker spot, but was different from both xanthine and hypoxanthine markers. Similar chromatographic evidence was obtained when propan-1-ol–NH_3 was the developing solvent. These findings were taken to indicate that the products of hydrolysis of IMP were inosine and inorganic phosphate. Chromatograms also showed that complete hydrolysis of the IMP by the enzyme preparation occurred after incubation for 4 hr. (see also Fig. 2).

Fig. 1. Rate of formation of ammonia from glutamine. Each vessel contained 4 ml. of reaction mixture, including 1·3 ml. of enzyme suspension, 0·008 M glutamine and 0·035 M-$NaHCO_3$; pH 7·4. △, No addition; ○, 0·004 M CP; ▲, 0·004 M IMP; ●, 0·004 M ITP.

Fig. 2. Rate of formation of orthophosphate. Each vessel contained 4 ml. of reaction mixture, including 1·3 ml. of enzyme suspension, 0·008 M glutamine and 0·035 M-$NaHCO_3$; pH 7·4. No addition resulted in no phosphate present. ●, 0·004 M CP; ○, 0·004 M IMP; ▲, 0·004 M ITP.

Further chromatographic examination with the *iso*butyric acid–NH_3 solvent showed that as the incubation proceeded glutamine decreased in amount and glutamic acid appeared.

Possibility of AMP being an intermediate in the reaction

Effect on adenylic deaminase activity of washing the brain particles. If AMP is an intermediate in the reaction, the activity of the adenylic deaminase present in the enzyme preparation must be sufficiently high to produce NH_3 from AMP at a rate equal to, or greater than, the rate of production from glutamine and ITP (or IMP and CP). To measure the rate of this formation of NH_3, the crude homogenate, the once-washed particles and the twice-washed particles, were each incubated with AMP (final concn. 0·004 M) under the test conditions. The results given in Table 2 show that the washing procedure reduced formation of NH_3 from AMP to negligible proportions. The rate and magnitude of production of NH_3 from a mixture of glutamine, IMP and CP, under the same conditions, were not affected.

Experiments with ^{32}P-labelled IMP

^{32}P Labelled IMP (0·004 M), CP (0·004 M), carrier AMP (0·004 M) and glutamine (0·008 M) were incubated with the suspension for 60 min. Samples of the reaction mixture at zero time and at 60 min. were run on chromatograms and the AMP areas cut out, wet-ashed and counted as described by Eggleston & Hems (1952). There was no increase with time of the radioactivity associated with the AMP area. This showed that IMP had not been aminated, although 16 μg. of NH_3/ml. had been formed.

If the hydrolysis of AMP was inhibited, any AMP formed from IMP and glutamine would accumulate and could be detected. Heppel & Hilmoe (1951) showed that low concentrations of NaF inhibited 5′-nucleotidase. Paper chromatography indicated that NaF at a final concentration

Table 2. *Effect of washing rat-brain particles on adenylic deaminase activity and production of ammonia from glutamine*

Volume of reaction mixture was 3 ml., including 1·3 ml. of enzyme suspension; AMP, IMP and CP, 0·004 M; $NaHCO_3$, 0·035 M; pH 7·4. Temperature of incubation, 30°.

Enzyme suspension	NH_3 (μg./ml.) produced in 60 min. from AMP	NH_3 (μg./ml.) produced in 60 min. from Glutamine + IMP + CP
Whole homogenate	12·1	20·6
Once-washed particles	3·0	19·8
Twice-washed particles	0·6	19·5

of 0·02 M inhibited the hydrolysis of AMP and IMP completely, under the test conditions. No increase in inorganic phosphate was detected even after incubation for 4 hr. The previous experiment was repeated with NaF added to a final concentration of 0·02 M. After incubation for 4 hr. there was no increase in radioactivity associated with the AMP area.

Effect of L-*glutamic acid on production of* NH_3 *from glutamine*

Krebs (1935) showed that production of NH_3 from glutamine by brain glutaminase was inhibited equally by addition of either L- or D-glutamic acid. This observation might explain the decline in the rate of production of NH_3, with time, described above. Table 3 shows that formation of NH_3 from glutamine in the presence of IMP, CP and the brain preparation is inhibited 97 % by L-glutamic acid.

Effect of phosphate and sulphate on production of NH_3 *from glutamine*

Greenstein & Leuthardt (1948) found that certain ions, especially phosphate and sulphate, activated the glutaminase of rat-brain homogenates.

The preparation of solutions of CP, IMP and ITP involved decomposition of the barium salts with an excess of sodium sulphate. Thus solutions of these substances always contain sulphate. During incubation IMP undergoes hydrolysis and yields phosphate. It was considered that the increased production of NH_3 during incubation of glutamine with IMP and CP might therefore be due to glutaminase activation by sulphate and phosphate.

To investigate this possibility, phosphate equivalent in amount to the IMP usually added was incubated with glutamine and the enzyme. A second vessel also contained sulphate (0·002 M final concn.). This amount was added to allow for sulphate that had been added for complete precipitation of barium from IMP and CP, and was about 17 % in excess. Table 4 shows the results of production of NH_3 in a typical experiment.

The figures show that both phosphate and sulphate stimulate production of NH_3 at low concentration and could explain the effect of addition of IMP and CP.

Effect of various ions on production of NH_3 *from glutamine*

Phosphate. A range of phosphate concentrations, 0·00125–0·08 M, was tested; the results in Fig. 3 show that formation of NH_3 increased with phosphate concentration and had not reached a maximum at 0·08 M.

The effect of glutamic acid on this stimulation was also tested. Two levels of phosphate concentration were used and incubated with glutamine and enzyme under the test conditions. At the same time a similar experiment was run where glutamic acid was also added at half of the concentration of the glutamine present. Fig. 4 shows that doubling the concentration of phosphate increased the initial rate of formation of NH_3, over the blank value, by a factor of 2·7. In the presence of the glutamic acid the rate of formation of NH_3 was much reduced. In the absence of added inorganic phosphate glutamic acid inhibited production of NH_3 completely.

Other ions. A variety of other ions was tested at a concentration of 0·013 M. The results are given in

Table 3. *Effect of glutamic acid on production of ammonia*

Volume of reaction mixture was 4 ml., including 1·3 ml. of enzyme suspension. IMP and CP, 0·004 M; glutamine, 0·008 M; L-glutamic acid, 0·004 M; $NaHCO_3$, 0·035 M; pH 7·4. Temperature of incubation, 30°.

	NH_3 produced in 60 min. (μg./ml.)
IMP + CP + glutamine	18·9
IMP + CP + glutamine + glutamic acid	0·6

Table 4. *Effect of phosphate and sulphate on production of ammonia from glutamine*

Volume of reaction mixture was 4 ml., including 1·3 ml. of enzyme suspension. IMP and CP, 0·004 M; Na_2HPO_4, 0·004 M; Na_2SO_4, 0·002 M; $NaHCO_3$, 0·035 M; pH 7·4. Temperature of incubation, 30°.

	NH_3 produced in 60 min. (μg./ml.)
IMP + CP + glutamine	15·9
CP + glutamine	10·6
PO_4^{3-} + SO_4^{2-} + glutamine	14·3
PO_4^{3-} + glutamine	9·4
Glutamine	2·9

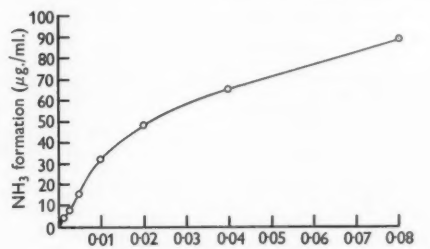

Fig. 3. Effect of increasing phosphate concentration on production of ammonia from glutamine. Each vessel contained 4 ml. of reaction mixture, including 1·3 ml. of enzyme suspension, 0·008 M glutamine, 0·035 M-$NaHCO_3$; pH 7·4. Phosphate concentrations varied as shown. Time of incubation, 60 min.

Table 5. Pyrophosphate caused an increase in production of NH_3 nearly double that caused by the same concentration of phosphate. The pyrophosphate was analysed by paper chromatography with formic acid–*iso*propyl ether as the developing solvent; no hydrolysis to inorganic phosphate occurred.

Soluble enzyme

Several attempts were made to prepare a soluble enzyme preparation. The suspension of rat-brain particles in either water or bicarbonate buffer (0·035 M) was shaken with ballotini beads in a Mickle vibrator for 15 min. in the cold room. The clear supernatant obtained on centrifuging was tested for enzyme activity under the conditions described above but was found inactive. The sediment was also inactive, as was the recombined supernatant and sediment.

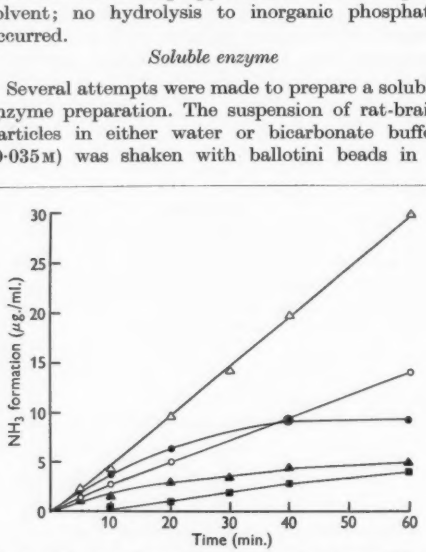

Fig. 4. Effect of L-glutamic acid on the rate of production of ammonia from glutamine in the presence of different concentrations of orthophosphate. Each vessel contained 4 ml. of reaction mixture, including 1·3 ml. of enzyme suspension, 0·008 M glutamine and 0·035 M-$NaHCO_3$; pH 7·4. ■, No additions; ○, 5 μmoles of phosphate/ml.; △, 10 μmoles of phosphate/ml.; 4 μmoles of L-glutamic acid resulted in no ammonia production; ▲, 4 μmoles of glutamic acid + 5 μmoles of phosphate/ml.; ●, 4 μmoles of glutamic acid + 10 μmoles of phosphate/ml.

Table 5. *Effect of various salts on glutaminase of rat brain*

Enzyme suspension, 1·3 ml. Substrate, glutamine. Concentration of salt, 0·013 M; HCO_3^-, 0·035 M; pH 7·4. Temperature of incubation, 30°.

Salt added	NH_3 formation (μg./ml./120 min.)
Nil	3·7
Na_2HPO_4	40·6
Na_2SO_4	23·6
$NaBO_3$	12·8
$Na_2S_2O_3$	16·3
KIO_3	5·5
$Na_4P_2O_7$	77·0
NaF	5·1
Na_2HAsO_4	14·7
$K_4Fe(CN)_6$	22·2
$K_3Fe(CN)_6$	1·1
Sodium ethylenediaminetetraacetate	8·3
$MgCl_2$	8·6

DISCUSSION

The incubation of IMP and CP or ITP (Table 1) with glutamine in the presence of washed rat-brain particles gives a greater production of ammonia than is found with glutamine alone. It has also been shown that the added glutamine is the primary source of the ammonia.

Washing the rat-brain particles resulted in a very marked decrease in ability to deaminate AMP (Table 2), whereas the rate and magnitude of production of ammonia from glutamine, CP and IMP under the same conditions was not affected. Further, when these twice-washed particles were incubated with glutamine, CP, ^{32}P-labelled IMP and carrier AMP there was no incorporation of radioactivity into the AMP, but production of ammonia was unaffected. In fact, no evidence for the amination of IMP to form AMP was found under conditions where IMP and CP stimulated the formation of ammonia from glutamine. This was taken to imply that the mechanism suggested by Weil-Malherbe (1953) for this stimulation, involving the intermediate amination of IMP, was not operative. Another explanation was sought.

Greenstein & Leuthardt (1948) had shown that rat-brain glutaminase was activated by very low concentrations of ions, especially phosphate and sulphate. Production of ammonia from glutamine under the test conditions could be explained satisfactorily in this manner.

The solution of ITP contained a little inorganic phosphate initially as can be seen from Fig. 2. It was found that both IMP and ITP were hydrolysed by the enzyme preparation to yield inorganic phosphate. The rate of hydrolysis of CP was very much slower.

The preparation of the solutions of the sodium salts of these compounds is achieved by precipitating the barium from solution with a slight excess of sodium sulphate, as a result of which these solutions always contain some sulphate.

The effect of inorganic phosphate and sulphate at low concentrations (0·004 M) was tested on the system deaminating glutamine; both were found to stimulate production of ammonia. The combined stimulation of these ions at this concentration was approximately equal to that of adding the same concentration of IMP and CP.

The effect of addition of glutamic acid to the enzyme system deaminating glutamine in the

presence of IMP and CP resulted in about 97% inhibition of production of ammonia. This result would be expected if the enzyme responsible were the brain glutaminase described by Krebs (1935). With inorganic phosphate (5 or 10 μmoles/ml.) replacing the IMP and CP in the system, addition of glutamic acid (4 μmoles/ml.) was accompanied by a very marked decrease in production of ammonia.

Greenstein & Leuthardt (1948) found that bicarbonate buffer did not activate glutaminase. Further evidence for this has been found in the low blank values. They measured the stimulatory effect of various ions on glutaminase, and some organic derivatives of phosphate and arsenate. The present study has included more multivalent ions, since Greenstein & Leuthardt (1948) had shown that univalent ions were poor stimulators of the reaction. It was concluded that, in general, as the ionic charge increased so did the effectiveness in stimulating production of ammonia (Table 5).

The effect obtained by Weil-Malherbe (1953) can be readily explained on the basis of an anion activation of brain glutaminase by phosphate formed by hydrolysis during the incubation, or by phosphate and sulphate added initially in these solutions.

SUMMARY

1. The formation of ammonia from glutamine by rat-brain particles in the presence of inosine triphosphate, or inosinic acid and creatine phosphate, is explained by the presence of low concentrations of phosphate and sulphate which activate the glutaminase present.

2. The reaction is 97% inhibited by the addition of L-glutamic acid.

3. The effect of various other ions on the reaction was studied. The results indicated that, as the ionic charge increases, so does the efficacy in stimulating production of ammonia.

4. The synthesis of adenosine 5′-monophosphate from inosinic acid does not occur in this system under these conditions.

I wish to thank Professor H. A. Krebs, F.R.S., and Dr G. Weber for their interest and advice in this work. I am also indebted to the Agricultural Research Council for the award of a grant for training in research methods.

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The Purification and Properties of Arginine Phosphokinase

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Phosphocreatine and phosphoarginine have been known for many years, but in spite of their importance in the process of muscular contraction little attention has been paid to the enzymes involved in their metabolism. The first detailed studies of creatine phosphokinase were made by Noda, Kuby & Lardy (1954), Kuby, Noda & Lardy (1954) and Ennor & Rosenberg (1954). The enzyme arginine phosphokinase was first found to be present in crude extracts of crab muscle by Lohmann (1935), who showed that it was responsible for catalysing the equilibrium as expressed by the reaction phosphoarginine + adenosine diphosphate $\rightleftharpoons$ arginine + adenosine triphosphate. The presence of the enzyme in extracts of octopus muscle and the jaw muscles of the echinoid *Sphaerechinus granularis* was demonstrated by Lohmann (1936) and by Baldwin & Needham (1937) respectively. Soreni, Dvornikova

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& Degtyar (1949) reported the isolation of the enzyme in a crystalline state following ammonium sulphate fractionation of aqueous extracts of the muscles of fresh-water crabs. These workers studied only the reverse reaction and gave but a brief description of the properties of the enzyme.

The inadequacy of methods hitherto available for the isolation of phosphoarginine and the estimation of arginine have precluded detailed studies of the properties of arginine phosphokinase. The isolation of phosphoarginine in good yield from crayfish muscle (Ennor, Morrison & Rosenberg, 1956) and the description of a sensitive and reliable procedure for the estimation of arginine (Rosenberg, Ennor & Morrison, 1956) have provided an opportunity for a detailed study of the enzyme, and this communication reports some results with a highly active preparation of the enzyme from the tail muscle of the sea crayfish (*Jasus verreauxi*).

In this paper the following abbreviations will be used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; PA, phosphoarginine; APK, arginine phosphokinase; EDTA, ethylenediaminetetraacetic acid; DNP, 2:4-dinitrophenol.

EXPERIMENTAL

Materials and methods

Phosphoarginine. PA was isolated from the tail muscle of the sea crayfish (*Jasus lalandii*) as described by Ennor *et al.* (1956). Solutions of the sodium salt were prepared by the careful addition of Na_2SO_4 (saturated solution) to a solution of the barium salt, and the end-point was judged by testing with sodium rhodizonate. The $BaSO_4$ was removed by centrifuging and washed several times with water. The washings were added to the original supernatant and the solution, after adjustment to pH 7·2 by the addition of N-HCl, was made up to volume. This method gave a quantitative recovery of PA and the resultant solution contained less than 0·2% of free arginine.

Adenosine triphosphate and adenosine diphosphate. Both were commercial samples (Sigma Chemical Co.) of the hydrated sodium salts of over 95% purity. Paper electrophoretic examination showed that the ATP contained traces of ADP, and the ADP contained traces of adenosine 5′-phosphate. The ATP also contained 0·3% of inorganic P. Solutions of ATP were initially pH 3·6 but were immediately adjusted to pH 8·5 by the addition of N-NaOH before making to volume. With ADP no such adjustment was necessary and the solutions were used at pH 7·6. The concentrations of both were checked by measurement of the absorption at 260 mμ. of dilutions in 0·1 N-HCl by assuming a molecular extinction value for adenosine 5′-phosphate of $14{\cdot}9 \times 10^3$.

Adenylic acid. Adenosine 5′-phosphate was prepared from ATP by the action of apyrase and was purified as described by Kerr (1941). It was obtained as the crystalline free acid and was stored as such. Solutions were made up as required and adjusted to pH 7·4 by the addition of N-NaOH.

Inosine triphosphate. This was prepared from ATP by the method of Kleinzeller (1942).

Arginine. A commercial sample (British Drug Houses Ltd.) of L-arginine monohydrochloride was used without recrystallization. The purity was checked by N analysis. Solutions were made up as required and adjusted to pH 8·5 by the addition of N-NaOH.

Other guanidino compounds. Creatine and glycocyamine were obtained from British Drug Houses Ltd. L-Canavanine sulphate was obtained from the California Foundation for Biochemical Research. Agmatine sulphate was obtained from Light and Co. Ltd. These compounds were used without recrystallization. Argininic acid was synthesized in poor yield by the method of Felix & Schneider (1938). α-Carbamidoarginine was synthesized by the method of Boon & Robson (1935). L-Homoarginine was synthesized by the method of Stevens & Bush (1950). Arginine methyl ester dihydrochloride was synthesized from arginine monohydrochloride by the method of Fischer & Suzuki (1905). Negmine (*N*-ethylglycocyamine) was a gift from Dr Armstrong of the University of Utah, U.S.A. Taurocyamine was prepared by the guanylation of taurine with *S*-ethylthiourea hydrobromide, as described by Brand & Brand (1942). β-Guanidopropionic acid and δ-guanido-*n*-valeric acid were prepared by the guanylation of β-alanine and δ-amino-*n*-valeric acid respectively with *O*-methyl*iso*urea hydrochloride, according to the general method of Schutte (1943). The δ-amino-*n*-valeric acid used was prepared from *cyclo*pentanone by the following reaction sequence: *cyclo*pentanone (1) ⟶ *cyclo*pentanone oxime (2) ⟶ α-piperidone (3) ⟶ δ-amino-*n*-valeric acid HCl. Reaction (1) was carried out by the procedure described by Bousquet (1943) for the synthesis of heptaldoxime. Reactions (2) and (3) were carried out as described by Schniepp & Marvel (1935).

All compounds were recrystallized twice before use, and were pure as judged by the melting point and one-dimensional paper chromatography in propanol–ammonia–water (60:30:10).

Solutions of PA, ATP, ADP, adenosine 5′-phosphate and arginine were stored at −15°.

Buffer systems. *N*-Ethylmorpholine (Eastman Kodak) was redistilled under reduced pressure before use. The components of other buffer systems were products of British Drug Houses Ltd.

Inhibitors. *p*-Chloromercuribenzoic acid was a Sigma Chemical Co. product. Diphenylchloroarsine was a gift from Sir Rudolph Peters, F.R.S. *o*-Iodosobenzoic acid and *N*-ethylmaleimide were gifts from Drs K. Pausacker and F. Hird of the University of Melbourne. Other inhibitors were products of British Drug Houses Ltd.

Estimation of protein

Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill & David (1949), crystalline ox-serum albumin being used as the standard.

Determination of enzymic activity

Unless otherwise stated, the following techniques were used to determine the activity of the enzyme:

Forward reaction: PA + ADP → Arginine + ATP. To 10 ml. graduated test tubes was added 0·8 ml. of a stock solution containing 0·1 ml. of 0·5 M *N*-ethylmorpholine (pH 7·2), 0·1 ml. of 0·05 M PA, 0·1 ml. of 0·1 M-$MgSO_4$, 0·1 ml. of 0·01 M ADP and 0·4 ml. of water. The tubes were

equilibrated in a water bath at 5° and the reaction was started by the addition of 0·2 ml. of APK solution. The APK solution was diluted to the required concentration in buffer (0·05 M, pH 7·2); this gave a final buffer concentration of 0·06 M. At the end of the incubation period the reaction was stopped by the addition of 1·0 ml. of an EDTA–NaOH mixture containing 0·4 ml. of 0·2 M EDTA (adjusted to pH 7·6) and 0·6 ml. of 5 N-NaOH. The arginine released in the reaction was estimated in the same tubes by the method of Rosenberg *et al.* (1956) without any further addition of NaOH. Under these conditions, the reaction followed zero-order kinetics.

Reverse reaction: Arginine + ATP → PA + ADP. The velocity of the reverse reaction was determined by acid hydrolysis of the PA formed and estimation of the inorganic P released. To 10 ml. graduated test tubes was added 0·8 ml. of a stock solution containing 0·1 ml. of 0·1 M arginine, 0·1 ml. of 0·05 M ATP, 0·1 ml. of 0·1 M-$MgSO_4$, 0·1 ml. of 0·5 M *N*-ethylmorpholine (pH 8·4) and 0·4 ml. of water. The tubes were equilibrated in a water bath at 5° and the reaction was started by the addition of 0·2 ml. of APK solution. The enzyme was diluted in *N*-ethylmorpholine (0·05 M, pH 8·4), making the final buffer concentration 0·06 M. The reaction was stopped by the addition of 1·0 ml. of 0·2 N trichloroacetic acid, the tubes were removed from the bath and placed in boiling water for 1 min. (Under these conditions PA is quantitatively hydrolysed to arginine and inorganic P.) They were then rapidly cooled by agitation in an ice bath and the inorganic P was estimated by a modification of the method of King (1932). The reagents [2·0 ml. of 5% (w/v) ammonium molybdate in 15% (v/v) H_2SO_4 and 0·5 ml. of the reducing agent] were added to each tube at half-minute intervals. The volume was adjusted to 10·0 ml. by the addition of water and the contents were mixed. Colour intensities were measured at 30 sec. intervals, 10 min. after the addition of the above reagents. (It should be noted that concentrations of *N*-ethylmorpholine greater than 0·08 M interfere with the determination of inorganic P.)

When the reaction velocity is determined by the estimation of inorganic P arising from PA as a result of hydrolysis in trichloroacetic acid, allowance has to be made for the inorganic P present in the ATP and that released from ATP as a result of hydrolysis in 0·1 N trichloroacetic acid at 100° and in acid molybdate during colour development. The correction may be determined in one of two ways, both of which should give the same value: (i) the complete system is stopped at zero time with trichloroacetic acid, the mixture heated at 100°, the colour developed and the extinction value determined at a fixed time after colour development. The value so obtained is then subtracted from the extinction values obtained after a similar treatment of the reaction system following incubation. Alternatively, (ii) the reaction is carried out for various time periods and treated as described above. The slope of the line obtained as a result of the extrapolation to zero time of a plot of the extinction values against reaction time gives a measure of the reaction velocity. The correction value is given by the point where the line cuts the ordinate. Experimentally it has been found that method (ii) gives a value which is about 10% higher than that obtained by method (i), and it seems likely that this is the correct zero-time value; for when this correction is used the reaction rate is proportional to the enzyme concentration. For this reason method (ii) has been used throughout this work, although no explanation can be offered for the difference mentioned. Careful attention was paid to the development time and hydrolysis conditions.

Arbitrary units of APK activity. One unit of APK activity, in the forward reaction, is defined as the amount of enzyme which releases 1 μmole of arginine from PA in 1 min. at pH 7·2 and at 5°.

Specific activity is defined as the number of units of APK/mg. of protein.

RESULTS

Preparation of arginine phosphokinase

Sea crayfish were used as the source of APK because of the large muscle mass and because they were available throughout the year. Attempts made to isolate the enzyme from this source by the method developed by Soreni *et al.* 1949 for fresh-water crab muscle were unsuccessful, and it was found that the fractionation pattern for APK from sea-crayfish muscle differed considerably from that for APK from fresh-water crab muscle. Therefore, attention was turned to the development of a method for the isolation of the enzyme from sea-crayfish muscle. This method is given below.

Extraction of tissue. Live crayfish (*Jasus verreauxi*) were obtained from sea-water storage pens and transported to the laboratory in bags packed with wet seaweed. All specimens arrived in good condition. From this stage onwards, all operations were carried out in the cold room at 5°. The tail muscles of six live crayfish were rapidly excised and passed twice through a mincer. A sample (500 g.) of the minced muscle was extracted by stirring with 3 vol. of water for 30 min. and then centrifuged for 15 min. at 1000 *g* and 0°. The product did not sediment well and it was necessary to pour off carefully the opalescent uppermost layer from the middle gelatinous layer. The two bottom layers were extracted with 1·5 vol. of water (750 ml.) by stirring for 30 min., and again centrifuged at 1000 *g* for 15 min. at 0°. The opalescent supernatant was poured off from the firmly packed residue and combined with the first extract. The pH of the combined extracts was 6·6. Both these extracts were of the same specific activity, and further quantities of the enzyme, also of the same specific activity, could be obtained as a result of a third extraction with 1·5 vol. of water. This extract contained only a small amount of protein and was discarded.

First ammonium sulphate fractionation. For convenience, two 500 ml. samples of the extract were taken and fractionated simultaneously. Solid $(NH_4)_2SO_4$ (31·5 g./100 ml.) was added slowly to each sample with mechanical stirring. The precipitate was removed by centrifuging at 5000 *g* for 10 min. at 0° and discarded. To the clear, colourless supernatant solid $(NH_4)_2SO_4$ (10·5 g./100 ml. of original extract) was added slowly with mechanical stirring. A grey precipitate was removed by centrifuging at 5000 *g* for 10 min. at 0° from a clear, colourless supernatant, which was discarded. The two precipitates were each dissolved in 100 ml. of water and combined.

Second ammonium sulphate fractionation. Ammonia soln. (17 N) was added to a saturated solution of $(NH_4)_2SO_4$ at 5° so that (on testing a dilution of 1 in 10) the pH was 8·2. This solution was added dropwise with mechanical stirring to the

enzyme solution obtained from the previous step until 0·7 saturation was reached. The grey precipitate was removed by centrifuging at 5000 *g* for 10 min. at 0° and discarded. The supernatant was then brought to 0·8 saturation by the further addition of the alkaline $(NH_4)_2SO_4$ solution. The suspension was centrifuged at 5000 *g* for 15 min. at 0°, the supernatant discarded and the white precipitate dissolved in water to give a final volume of 50 ml.

Attempts to increase the specific activity of this enzyme preparation were unsuccessful. Low-temperature fractionation with both ethanol and propanol at pH 6·6 and 8·2 in the presence and absence of magnesium led to marked loss of activity. The enzyme was not precipitated by $MgSO_4$.

Although APK constituted the major portion of the final product, it could not be induced to crystallize. The addition of saturated $(NH_4)_2SO_4$ solutions of varying pH in amounts just insufficient to cause precipitation gave rise to amorphous precipitates only.

The summary of the yields and degrees of purification of APK (Table 1) obtained with a typical preparation indicated that this fractionation procedure gives approximately a threefold increase in the purity of the enzyme with an overall recovery of 21 %.

Tests for the presence of other enzymes in the APK preparation

Tests for the presence of phosphoamidase, ATPase and myokinase activities in the APK preparation were carried out in the forward reaction system with the enzyme at a concentration 1000 times greater than that used in the standard-activity determination. Phosphoamidase activity was tested for at pH 7·2 and 8·4 by determination of any arginine released from PA in the absence of ADP; no such activity was detected. When ADP was replaced by ATP there was again no release of arginine, indicating the absence of a Mg^{2+} ion-activated ATPase, which conclusion is confirmed by the fact that there was no release of inorganic P when the enzyme was incubated with ATP. The replacement of ADP by ATP and adenosine 5′-phosphate led to the release of arginine. Thus an enzyme with myokinase-like activity was a contaminant of the preparation; but it was concluded that this was a minor contaminant for when tests were made at the usual level of 1 μg. of protein/ml. no trace of activity could be detected.

Electrophoretic analysis

The electrophoretic pattern of the final preparation of APK is illustrated in Fig. 1. All the components migrated to the anode at pH 8·6. An attempt was made to carry out a second run in acetate buffer (pH 5·7), but on dialysis of the enzyme against the buffer before electrophoresis, there was a marked precipitation of protein. It was not possible to redissolve the precipitate by dialysis against buffers with higher pH values. From a study of the effects of pH on the enzymic activity and protein content of the APK solution the enzyme appears to have an isoelectric point near pH 5. Because of the rapid denaturation of the enzyme at pH values below 5·7, it was not possible to carry out an electrophoretic run at pH values where the protein would be positively charged. It was calculated that at pH 8·6 the major component consti-

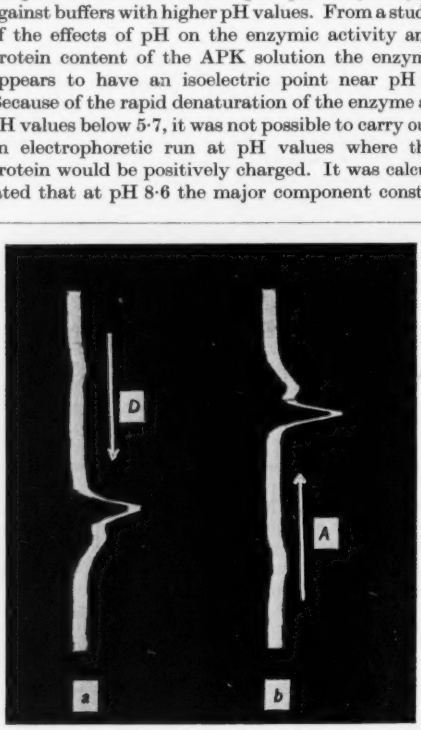

Fig. 1. Electrophoretic patterns of the APK preparation obtained after fractionation as described in the text. Veronal buffer, pH 8·6; ionic strength, 0·1; temperature, 1°; field strength, 6·6 v/cm.; protein concentration, 1%; time, 272 min. *a*, Descending boundary; *b*, ascending boundary.

Table 1. *Summary of yields and specific activities of fractions obtained during the fractionation of APK*

Wt. of muscle, 500 g. Details are described in the text.

Fraction	Volume (ml.)	Total protein (g.)	Total units	Specific activity	Recovery (%)
Extract	2050	22·1	530 000	24	(100)
First ammonium sulphate ppt.	570	8·3	282 000	34	53
Second ammonium sulphate ppt.	54	1·7	110 000	67	21

tuted 67 % of the total protein and had a mobility of $-2{\cdot}84 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$.

In order to determine whether or not the main component was responsible for the APK activity, the protein solution was dialysed against 0·04 M phosphate buffer (pH 7·4) and subjected to paper electrophoresis. Fig. 2 shows that staining with bromophenol blue (personal communication from Dr W. E. van Heyningen, 1954) indicates the presence of a major component and a faster-moving minor component. From a second paper strip run at the same time areas corresponding to the stained sections were excised and added to the standard-test system for the forward reaction. The results showed that the major, slower-moving component possessed APK activity, whereas the faster-moving component showed none. The components showed neither myokinase nor phosphoamidase activity.

Elution of the stained protein bands as described by Durrum (1955) and subsequent determination of the optical density indicated that APK constituted 80 % of the total protein. This compares with a value of 67 % obtained from the run in the Tiselius electrophoresis apparatus.

The fact that a preparation of high purity was obtained with only a threefold increase in specific activity indicates that APK forms a high proportion of the protein of the muscle extract. This is also indicated by the finding that only four components could be detected in the extract by paper electrophoresis.

General properties of APK

The APK preparation could be dialysed against water and *N*-ethylmorpholine and phosphate buffers (0·05 M, pH 7·2) without loss of enzymic activity. There was no loss of activity when the protein solution was freeze-dried after dialysis against phosphate buffer, but after dialysis against water freeze-drying led to an almost complete loss of activity. When water was added to the water-dialysed, freeze-dried protein the solution was opalescent and the pH was 4·4; this low pH presumably accounts for the loss of activity. Freezing and thawing (four times) of a 1 % (w/v) aqueous solution of the enzyme (pH 7) produced no loss of activity. Although the enzyme could be dialysed against phosphate buffer and freeze-dried, it was more convenient to store the solution at 5° in the presence of an equal volume of glycerol. This dilution was made after determination of the protein content by the biuret method to avoid interference by the glycerol. Suitable reaction rates were obtained with high dilutions of the glycerol solution so that the concentrations of glycerol and $(NH_4)_2SO_4$ in the final reaction mixture were such that they had no effect on the activity of the enzyme.

The initial specific activity of all APK preparations (67–80) fell during the first 2–3 days of storage to a value of 46. This decrease in activity was independent of the method of storage but after its occurrence the specific activity of the glycerol solutions of APK remained constant for at least 12 months. In the absence of glycerol further changes occurred after the first 2–3 days: thus it was found that aqueous and $(NH_4)_2SO_4$ solutions of the enzyme became opalescent within a week and a precipitate settled on the walls of the vessel. There was, however, no fall in the specific activity of the supernatant. On the other hand, glycerol solutions of the enzyme remained clear.

Effect of temperature and dilution on APK activity. The initial experiments designed to determine APK activity in the forward reaction were carried out at 30° but the results were unreliable. This was at first believed to be due to the effect of temperature on the enzyme, and an incubation temperature of 5° was then chosen. However, even at this temperature it was found that the observed specific activity varied with the degree to which the enzyme was diluted before the addition to the test medium. Thus

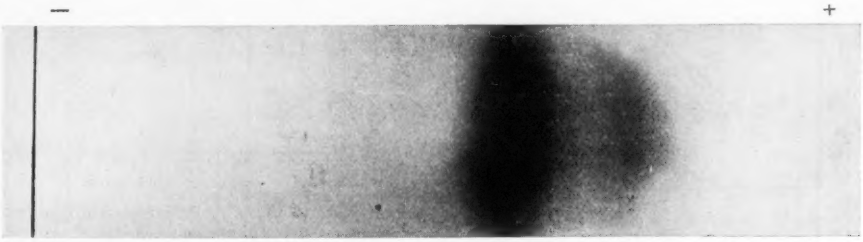

Fig. 2. Separation by paper electrophoresis of the proteins of the APK preparation obtained as described in the text. Phosphate buffer (0·04 M, pH 7·4) containing 5 % (v/v) ethylene glycol; current, 8 mA; time, 22 hr.; temperature, 22°. *a*, APK; *b*, contaminating protein.

a large volume of highly diluted enzyme was not as active as a small volume of a correspondingly more concentrated enzyme, even though the reaction rate was constant in all cases. This indicates that there is no inactivation of APK as a result of enzyme dilution in the presence of substrate; inactivation therefore occurs as a result of the initial dilution of the enzyme with buffer. This effect was most marked when the concentration of protein in the diluted enzyme solution was less than 2·5 μg./ml. and absent at concentrations greater than 5 μg./ml. This inactivation of the enzyme at high dilutions was apparently not due to oxidation of –SH groups, for the effect was still observed even when the enzyme was diluted in the presence of cysteine. A temperature of 5° was chosen for all subsequent work as the reaction rate was slowed sufficiently to permit the use of larger amounts of enzyme and thus to avoid inactivation of the type referred to above.

Effect of enzyme concentration on the reaction velocity. The initial velocity is proportional to the enzyme concentration in both the forward and reverse reactions over the concentration range studied (Figs. 3, 4). It will be noted that the velocity of the forward reaction is about five times as fast as the reverse reaction.

Effect of pH *on the reaction velocity.* The relationship between pH and reaction velocity in the forward reaction (Fig. 5) indicates that maximum velocity is reached at pH 6·8 in phosphate, at

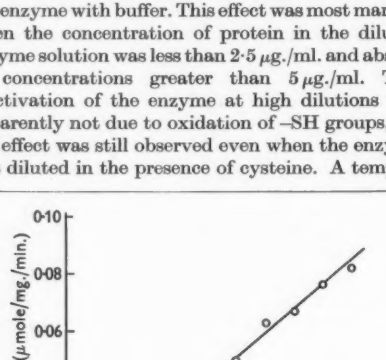

Fig. 3. Effect of enzyme concentration on the reaction velocity of the forward reaction at pH 7·2. Conditions as described in the text, except that various volumes of a stock enzyme solution (5 μg. of protein/ml.) were added. Reaction time, 4 min.; temperature, 5°.

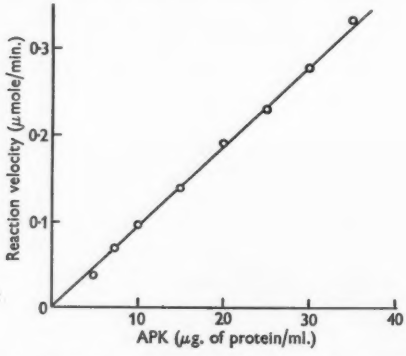

Fig. 4. Effect of enzyme concentration on the reaction velocity of the reverse reaction at pH 8·4. Conditions as described in the text, except that various volumes of a stock enzyme solution (100 μg. of protein/ml.) were added. Reaction time, 3, 6 and 9 min.; temperature, 5°.

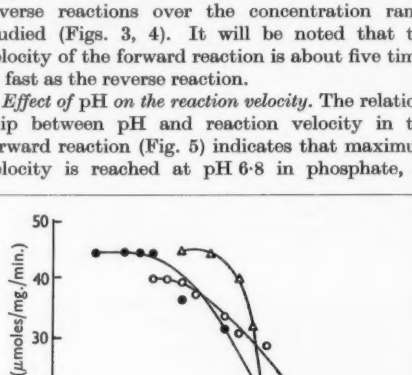

Fig. 5. Effect of pH on the initial reaction velocity of the forward reaction. The system was as described in the text. The enzyme concentration was 1 μg. of protein/ml. and reactions were run for 3 and 6 min. The pH was determined with a glass electrode in a duplicate set of tubes. Temperature, 5°. ○, *N*-Ethylmorpholine (0·06 M); ●, phosphate (0·06 M); △, glycerophosphate (0·06 M).

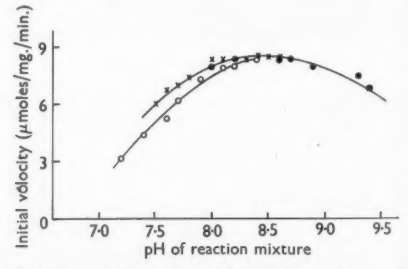

Fig. 6. Effect of pH on the initial reaction velocity of the reverse reaction. The system was as described in the text. The enzyme concentration was 15 μg. of protein/ml. and reactions were run for 5, 10 and 15 min. The pH was determined with a glass electrode in a duplicate set of tubes. Temperature, 5°. ○, *N*-Ethylmorpholine (0·06 M); ●, glycine (0·06 M); ×, glycylglycine (0·06 M).

pH 7·2 in glycerophosphate and at pH 7·0 in *N*-ethylmorpholine. At the pH optimum, the reaction rates were the same in phosphate and glycerophosphate, but were slightly lower in *N*-ethylmorpholine. Although the pH optimum in *N*-ethylmorpholine is at pH 7·0, further experiments on the forward reaction in this buffer were carried out at pH 7·2, as better buffering capacity was obtained at this pH. Aminotrishydroxymethylmethane was not used as a buffer because it interferes with the estimation of arginine (cf. Rosenberg *et al.* 1956).

In the reverse reaction (Fig. 6) the pH–activity curves show that the optimum pH is about 8·4 in *N*-ethylmorpholine, glycine and glycylglycine buffers. At this pH the reaction velocity is the same in each of the three buffers.

Activation of APK by bivalent cations. The purified APK preparation shows only a small residual activity in the absence of bivalent cations (Fig. 7). The activity of the enzyme in the forward reaction is markedly increased by the addition of Mg^{2+} ions but Ca^{2+} ions are without effect. Although Mn^{2+} ions also activated the enzyme, these ions interfere with the arginine estimation by the formation of coloured ions in alkaline solution and so it was not possible to obtain a precise figure for the degree of activation. In the reverse reaction (Fig. 8) it was possible to demonstrate activation of the enzyme by both Mg^{2+} and Mn^{2+} ions, but here again there was no activation by Ca^{2+} ions. There was no activation of APK by Ba^{2+}, Sr^{2+}, Ni^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Fe^{3+} and Al^{3+} ions as tested in the reverse reaction.

The activity of the APK preparation in the absence of added ions (residual activity) was not removed by dialysis against 0·01 M EDTA (pH 7·2) for 18 hr., followed by dialysis against water. However, when EDTA (0·01 M) was added to the test medium there was a complete loss of residual activity.

Effect of inhibitors on APK activity. The effect of inhibitors on APK activity in the forward reaction is summarized in Table 2. The inhibition by iodoacetate, *p*-chloromercuribenzoate, *o*-iodosobenzoate, *N*-ethylmaleimide and diphenylchloroarsine characterizes APK as an –SH enzyme. The enzyme is very sensitive to *p*-chloromercuribenzoate, 100 % inhibition being obtained at 10^{-7} M. The high affinity of the enzyme for Mg^{2+} ions is indicated by the fact that an EDTA:Mg ratio of 2:1 is required for complete inhibition at pH 7·2. The inhibition by DNP is unusual, as this compound has not been shown to inhibit transphosphorylation at the substrate level. The nature of the inhibition was further investigated and it was found that when tests were made on the reverse reaction at pH 8·4 and pH 7·2 no inhibition resulted. A similar result was obtained with tests on the forward reaction at pH 8·4. It thus seems that DNP has no effect on transphosphorylation; its inhibitory action on the forward reaction at pH 7·2 may therefore be due to interaction with one of the substrates.

Specificity of APK. The ability of APK to phosphorylate other guanidine compounds was determined in the reverse reaction by estimating both the disappearance of free guanidine and the formation of acid-labile P. With canavanine it was possible to determine only the formation of acid-labile P, as

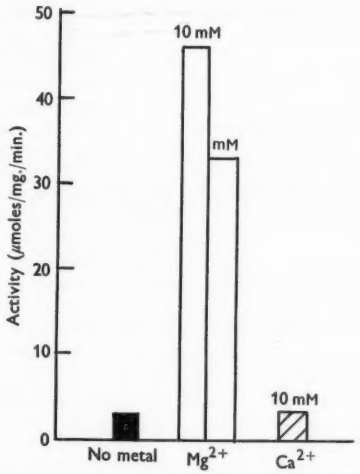

Fig. 7. Activation of APK by bivalent metals as determined in the forward reaction. Conditions as described in the text. Temperature, 5°.

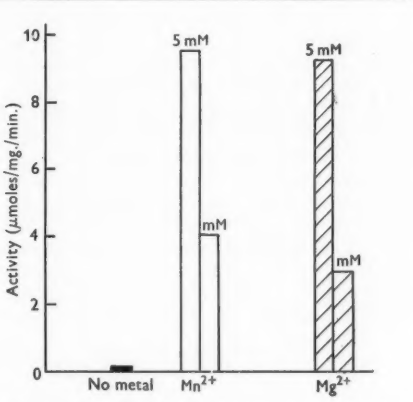

Fig. 8. Activation of APK by bivalent metals as determined in the reverse reaction. Conditions as described in the text. Temperature, 5°.

this compound does not give the guanidine colour reaction with α-naphthol and diacetyl. It can be seen (Table 3) that besides arginine there was also phosphorylation of arginine methyl ester, L-homoarginine and L-canavanine. However, the degree to which these compounds were phosphorylated was less than with arginine. There was no phosphorylation of agmatine, creatine, glycocyamine, taurocyamine, negmine, citrulline, argininic acid, δ-guanido-*n*-valeric acid, β-guanidopropionic acid or α-carbamidoarginine.

These latter compounds as well as canavanine and homoarginine had no effect on the rate at which arginine was phosphorylated when present in equimolar concentrations. Under the standard-test conditions arginine methyl ester was phosphorylated at the same rate as arginine, whereas the rates of phosphorylation of homoarginine and canavanine were negligible.

The enzyme is specific for ADP in the forward reaction; adenosine 5′-phosphate does not act as a phosphate acceptor. In the reverse reaction, ATP could not be replaced by inosine triphosphate or ADP.

Isolation of APK from the tail muscle of Cherix albidus

Specimens of fresh-water crayfish (*Cherix albidus* Clark) are available only in the summer months. On completion of the work on APK isolated from sea-water crayfish an attempt was made to isolate the enzyme from the tail muscle of *Cherix albidus* by the procedure described by Soreni *et al.* (1949).

The precipitate formed by the addition of $(NH_4)_2SO_4$ (43·2 g./100 ml.) to the extract was removed by filtration and the filtrate allowed to stand for 48 hr. at 5°. No crystalline material was formed but the amorphous precipitate which appeared after 18 hr. contained large amounts of APK of high specific activity (90). Electrophoretic analysis of this fraction in veronal buffer ($\mu = 0{\cdot}1$) at pH 8·6 showed the presence of three components, and it was estimated that APK constituted about 50 % of the total protein present.

Table 2. *Effect of various substances on APK activity in the forward reaction*

The enzyme (1 μg./ml.) was incubated for 5 min. in the presence of the inhibitor, *N*-ethylmorpholine (0·06 M, pH 7·2), PA (5 mM) and Mg^{2+} ions (5 mM). The reaction was started by the addition of ADP (mM) and stopped after 5 min. Total volume, 1·0 ml.; temp. 5°.

Compound	Concentration (M)	Inhibition (%)
Iodoacetate	1×10^{-2}	92
	1×10^{-3}	19
p-Chloromercuribenzoate	1×10^{-7}	100
	1×10^{-8}	27
o-Iodosobenzoate	1×10^{-3}	100
	1×10^{-4}	67
	1×10^{-5}	19
N-Ethylmaleimide	1×10^{-4}	85
	1×10^{-5}	10
Diphenylchloroarsine	1×10^{-4}	55
	1×10^{-5}	12
Chloroacetophenone	1×10^{-4}	0
Arsenite	1×10^{-3}	0
Ethylenediaminetetraacetic acid	2×10^{-2}	100
	1×10^{-2}	95
	5×10^{-3}	32
Dinitrophenol	1×10^{-3}	95
	1×10^{-4}	39

Table 3. *Specificity of APK: phosphorylation of guanidines*

The reaction mixture contained guanidine (2 mM), ATP (5 mM), Mg^{2+} ions (mM) and *N*-ethylmorpholine (0·06 M, pH 8·4). The enzyme (final concn., 46 μg. of protein/ml.) was added and the tubes were incubated for 17 hr. at 5°. Total volume, 1·0 ml.

Guanidine	Phosphorylation (%) (*a*)*	(*b*)†
L-Arginine	88	91
L-Arginine methyl ester	65	67
L-Canavanine	—	19
L-Homoarginine	23	26

* (*a*) Calculated on basis of the free guanidine remaining.
† (*b*) Calculated by determination of the guanidino P formed.

Table 4. *Spectrographic analysis of APK preparations*

(*a*) Figures represent a visual assessment of the concentration of each metal on an ash basis.

	>10%	1–10%	0·1–1%	<0·1%
Sea crayfish	Na, Al, Ca, P	Cu, Si, Mg, Fe	Mn	B
Fresh-water crayfish	Si, Al, Ca, P	Fe	Cu, Mg, Pb	Cd, Mn, Na

(*b*) Comparison of the relative concentrations (in arbitrary units) of metals present in the ash of each preparation as determined from the spectrograms.

Sample	Ash (%)	Na	Cu	Al	Si	Fe	Mg	Ca	P	Mn
Sea crayfish	2·2	10	10	10	4	5	10	10	10	10
Fresh-water crayfish	0·22	0·05	7	10	10	10	6	7	10	0·5

The only experiments carried out with this APK preparation indicated that both Mg^{2+} and Mn^{2+} ions markedly activated the enzyme and that Ca^{2+} ions were without effect.

Spectrographic analyses of APK preparations from fresh-water and sea crayfish

APK preparations from fresh-water and sea crayfish were dialysed against nine changes of water at 5° for three days and freeze-dried. The dry material was ashed and examined by arc spectrography.

The results (Table 4) of visual examination of the spectrograms indicated a much higher content of Mg^{2+} ions than of Mn^{2+} ions in both preparations. It is also of interest to note that both samples contained comparatively large amounts of Ca^{2+} ions, an ion which is incapable of activating the enzyme.

DISCUSSION

In the present work the major portion of the protein possessing APK activity was precipitated from the crude extract of sea-crayfish muscle in the fraction obtained between the addition of 31·5 and 42 g. of ammonium sulphate/100 ml.; the enzyme remaining in the supernatant was of much lower specific activity. This result differs sharply from that of Soreni *et al.* (1949), who isolated crystals of APK from the supernatant obtained after the addition of 43·2 g. of ammonium sulphate/100 ml. In one respect, however, the present results confirm those of these workers: diamond-shaped protein crystals were isolated from the supernatant (43·2 g. of ammonium sulphate/100 ml.) after standing for 24 hr. in the cold room at 5°. These were identical in appearance with those (designated crystals *B*) photographed by Soreni *et al.* (1949), who did not identify them as possessing enzymic activity. Preliminary results have indicated that they possess glyceraldehyde-phosphate dehydrogenase activity, and on examination in the Perkin–Elmer electrophoresis apparatus at pH 8·6 only one component was present.

An examination of fresh-water crayfish also failed to confirm the findings of Soreni *et al.* (1949), but it is of interest to note that whereas the fractionation pattern was different from that described by these workers it was also different from that observed in the sea-crayfish muscle. It is possible therefore that these differences may be due to species variation, but in any event it is apparent that the fractionation procedure described by Soreni *et al.* (1949) cannot be applied to the isolation of APK in crystalline form from the tail muscles of either sea-water or fresh-water crayfish available for the present work.

Differences in the properties of the APK described by Soreni *et al.* (1949) and those of our preparation are of a major nature. Thus the present results show that there is no loss of activity or of protein following dialysis against water and that the enzyme as isolated shows only slight activity in the absence of Mg^{2+} or Mn^{2+} ions. Soreni *et al.* (1949) have found that protein was precipitated when the enzyme was dialysed against water and that there was no separation of a cofactor after dialysis against a solution of ammonium sulphate. As no bivalent ions were added in the activity experiments it must be assumed that the enzyme was working at maximal activity, owing perhaps to the presence of Mn^{2+} ions bound to the enzyme. However, the analytical data, showing Mn^{2+} ion content of 0·27 %, refer only to mixed crystals of APK and an unknown protein. On the other hand spectrographic analysis of both the fresh-water and sea crayfish showed that Mg^{2+} ions were present in higher concentrations than Mn^{2+} ions. It is difficult to calculate the specific activity of the preparation described by Soreni *et al.* (1949) because the reaction rates were neither proportional to the enzyme concentrations nor linear with time. However, on the basis of an average value of the reaction rates at 30° the specific activity may be calculated to be approximately 1·2 at 5°. This figure may be compared with a specific activity of 9·2 at 5° obtained in the present work.

An ill-defined pH optimum for the reverse reaction in glycine buffer was found to lie at pH 8·4 in the present work, whereas Soreni *et al.* (1949) have claimed a well-defined pH optimum, in the same buffer, at pH 9·1. Another difference between the present results and those of the above authors lies in the isoelectric points; thus in crayfish APK the isoelectric point lies at about pH 5·0, but an unusually low figure of 3·5 was reported for crab APK.

N-Ethylmorpholine was chosen as the buffer for general work on the assumption that the concentration of free metal ion would not be reduced by reaction with the buffer and that thus complication of kinetic studies (to be reported) would be avoided. It is noteworthy therefore that the APK activity in the reverse reaction at the pH optimum is the same in *N*-ethylmorpholine as in glycine and glycylglycine, which coordinate with Mg^{2+} ions (Monk, 1951). Moreover in the forward reaction the velocity of the enzymic reaction in *N*-ethylmorpholine is less than in phosphate and glycerophosphate buffers, which also form complexes with Mg^{2+} ions (Tabor & Hastings, 1943). It seems therefore that under the conditions used there is no effective reduction of the Mg^{2+} ion concentration by the buffers which are capable of forming complexes with it.

The inhibition of enzymic activity by three classes of –SH reagents indicates that APK is

dependent upon the presence of thiol groups for activity. The inhibition by iodoacetate characterizes the –SH group(s) of APK as 'freely reacting' according to the classification of thiol enzymes by Barron (1951). The inhibition by diphenylchloroarsine indicates that the enzyme forms a stable monothioarsenite, and the failure of arsenite to inhibit suggests that the thiol groups are not in sufficiently close proximity to form a stable As-ring (Peters, 1952). The inhibition of the reaction by DNP is of interest in occurring at the substrate rather than at the enzyme level. Doubtless a similar explanation would account for the inhibiting effect of 2:4-dinitro-*o*-cresol on creatine phosphokinase activity (Ennor & Rosenberg, 1954).

The experiments on the specificity of APK give some indication of the groups required for interaction of the enzyme and substrate. The failure of the enzyme to phosphorylate argininic acid, agmatine, δ-guanido-*n*-valeric acid and α-carbamidoarginine suggests that the α-amino group of arginine is an essential requirement of the substrate. The carboxyl group on the other hand is not essential, as evidenced by the fact that both arginine and arginine methyl ester were phosphorylated at the same rate. These results may be compared with those obtained with arginase (Greenberg, 1951), for which enzyme a free carboxyl group but not an α-amino group is essential. An intact guanido group is required, as evidenced by the failure of APK to phosphorylate citrulline. The fact that homoarginine and canavanine are phosphorylated indicates that the enzyme is not absolutely specific. However, the rate of phosphorylation of these compounds is very slow. APK is similar to arginase and argininosuccinase (Greenberg, 1951; Walker, 1953) in that there is little or no differentiation between guanido and guanidoxy groups.

A comparison of the present results with those obtained with creatine phosphokinase by Kuby *et al.* (1954), Ennor & Rosenberg (1954) and Rosenberg & Ennor (1955) shows that there is a similarity in the properties of the two enzymes. Both are –SH enzymes of the monothiol type; both are activated by Mg^{2+} and Mn^{2+} ions, although creatine phosphokinase is also activated by Ca^{2+} ions. The pH optimum for the forward reaction in each case is about pH 7 and for the reverse reaction is from pH 8·4 to 8·8. Under optimum conditions the velocity of each forward reaction is about six times as fast as the reverse reaction. This similarity is perhaps not surprising as the creatine phosphokinase–phosphocreatine system presumably plays the same role in the metabolism of vertebrate muscle as the arginine phosphokinase–phosphoarginine system plays in the metabolism of invertebrate muscle.

SUMMARY

1. A method is described for the isolation of a highly active preparation of arginine phosphokinase from the tail muscle of the sea crayfish (*Jasus verreauxi*). The purity was estimated to be 67–80 %.

2. The enzyme had a pH optimum of 6·6–7·0 and 8·4–8·5 in the forward and reverse reactions respectively, as expressed by the equation: Phosphoarginine + adenosine diphosphate ⇌ arginine + adenosine triphosphate.

3. Mn^{2+} and Mg^{2+} ions activated the enzyme in both the forward and reverse reactions. There was no activation by Ca^{2+} ions.

4. The –SH nature of the enzyme was established as a result of the inhibition by iodoacetate, *p*-chloromercuribenzoate, etc. There was also inhibition by ethylenediaminetetraacetic acid and 2:4-dinitrophenol.

5. The enzyme phosphorylated homoarginine and canavanine. There was no phosphorylation of agmatine, argininic acid, δ-guanido-*n*-valeric acid, α-carbamidoarginine, citrulline, creatine, negmine, glycocyamine, β-guanidopropionic acid or taurocyamine.

6. Adenosine 5′-phosphate could not act as a phosphate acceptor in the forward reaction. Inosine triphosphate or adenosine diphosphate could not replace adenosine triphosphate in the reverse reaction.

It is a pleasure to express our thanks to Mr F. Dempster of the Commonwealth Serum Laboratories, Melbourne, and to Mr R. Adams for carrying out the electrophoretic measurements. Our thanks are also due to Mr D. J. David, Division of Plant Industry, C.S.I.R.O., Canberra, for the spectrographic analyses and to Mr D. Maguire for skilled technical assistance.

Note added in proof.

After this paper was submitted for publication the attention of the authors was drawn by the Editors to a paper (Szorenyi, Elodi & Devenyi, 1956) in which the diamond-shaped crystals isolated from a crab-muscle extract were shown to be D-glyceraldehyde 3-phosphate dehydrogenase.

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A Study of the Kinetics of the Reactions Catalysed by Arginine Phosphokinase

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Some general properties of arginine phosphokinase (APK), which catalyses the approach to equilibrium of the reaction phosphoarginine + adenosine diphosphate ⇌ arginine + adenosine triphosphate have been described in a previous communication (Morrison, Griffiths & Ennor, 1957). Although the properties of the enzyme have also been studied to some extent by Lohmann (1935; 1936) and Soreni, Dvornikova & Degtyar (1949), no detailed study has been made of the kinetics of the reaction. This has been undertaken in the present work, which was also designed to elucidate the role of Mg^{2+} ions in the mechanism of the reaction. It has previously been reported that Mg^{2+} ions activate APK (Morrison *et al.* 1957), and it is well known that Mg^{2+} ions activate other phosphate-transferring enzymes (Lehninger, 1950). However, little work has been directed towards an understanding of the role of Mg^{2+} ions in phosphate-transfer reactions. The results reported in this communication indicate that Mg^{2+} ions react with APK to form an active Mg–APK complex which is responsible for catalysing the reaction as expressed above. In this paper the following abbreviations will be used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; PA, phosphoarginine; APK, arginine phosphokinase.

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EXPERIMENTAL

The materials and experimental methods, together with the preparation of the enzyme used in the present investigation, have been fully described in a previous publication (Morrison *et al.* 1957). The APK preparation had a purity of 67–80%. The enzyme was stored at 5° in the presence of an equal volume of glycerol and diluted with 0·05M *N*-ethylmorpholine of the appropriate pH before addition to the test system. Although the preparation was contaminated by myokinase, the activity of this enzyme was not detectable at the low enzyme concentrations and short reaction periods used for the kinetic studies. Even with the larger enzyme concentrations and longer incubation periods used for the equilibrium experiments, it appeared that the presence of myokinase did not influence the equilibrium values. The detailed descriptions of the experimental conditions are given in the legends to the figures and tables. All experiments have been carried out at 5° for reasons previously explained (Morrison *et al.* 1957). The K_m values represent a measure of the substrate concentration required for half-maximum velocity and are not necessarily true dissociation constants of the enzyme–substrate complexes.

The reactions, PA + ADP→arginine + ATP and arginine + ATP→PA + ADP are taken as the forward and reverse reactions respectively.

RESULTS

Effect of bivalent cations on the reaction velocity. The effect of Mg^{2+} ion concentration on the reaction velocity of the reverse and forward reactions is

shown in Figs. 1 and 2 respectively. In the reverse reaction (Fig. 1) it is seen that there is a relationship between the initial velocity and the ATP and Mg^{2+} ion concentrations. Thus for each concentration of ATP, a maximum velocity is reached when the Mg^{2+} ion concentration is approximately equal to that of ATP. The maximum velocity is also dependent upon the concentration of ATP. When the Mg^{2+} ions:ATP ratio exceeded 1:1, as a result of increasing the Mg^{2+} ion concentration, there was no decrease in the reaction velocity.

In the forward reaction at pH 7·2 (Fig. 2) maximum enzymic activity at the three levels of ADP was attained when the concentration of Mg^{2+} ions was greater than that of ADP. There was no relationship between the concentrations of ADP and Mg^{2+} ions as was the case in the reverse reaction, for the ratio of Mg:ADP at maximum enzymic activity varied from 3 to 8 according to the ADP concentration. When the Mg^{2+} ion concentration was increased considerably above that required for maximum velocity, there was a fall in the reaction rate which was dependent upon the ADP concentration; the fall was greater with lower concentrations of ADP.

Dissociation constants of APK for Mg^{2+} *and* Mn^{2+} *ions.* In order to elucidate the role of bivalent metals in the action of APK, the Mg^{2+} ion concentration was varied while the other components of

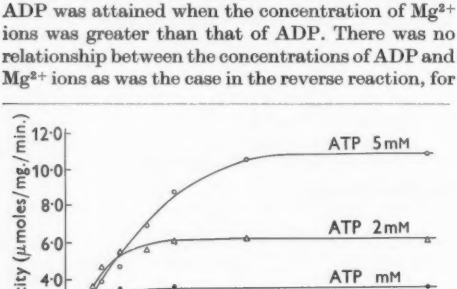

Fig. 1. Effect of Mg^{2+} ion concentration on the initial velocity of the reverse reaction in the presence of various concentrations of ATP. The reaction mixture contained arginine (10^{-2} M), *N*-ethylmorpholine (0·06 M, pH 8·4) and APK (25 μg. of protein/ml.). Total volume, 1·0 ml.; temp. 5°.

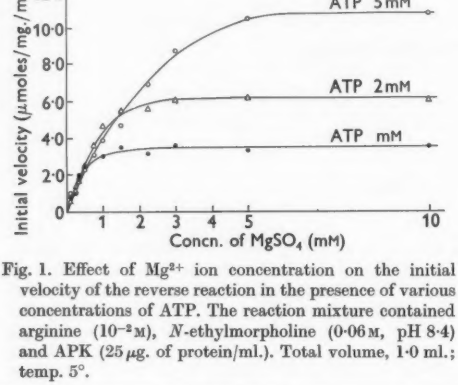

Fig. 2. Effect of Mg^{2+} ion concentration on the initial velocity of the forward reaction in the presence of various concentrations of ADP. The reaction mixture contained PA (5 mM), *N*-ethylmorpholine (0·06 M, pH 7·2) and APK (1 μg. of protein/ml.). Total volume, 1·0 ml.; temp. 5°.

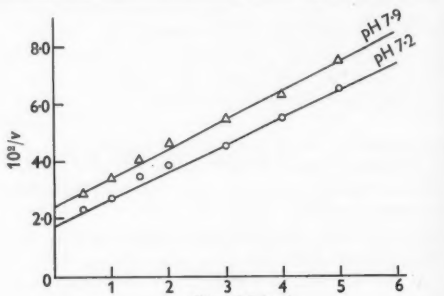

Fig. 3. Effect of Mg^{2+} ion concentration on the initial velocity of the forward reaction. The results are plotted according to the method of Lineweaver & Burk (1934). The conditions were the same as those described in Fig. 2. Concn. of ADP: mM. v = μmoles/mg./min.

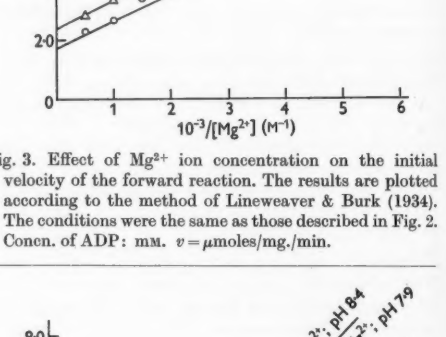

Fig. 4. Effect of Mg^{2+} and Mn^{2+} ion concentrations on the initial velocity of the reverse reaction. The results are plotted according to the method of Lineweaver & Burk (1934). The conditions were the same as those described in Fig. 1. Concn. of ATP: 5 mM. v = μmoles/mg./min.

both the forward and reverse reaction systems were held constant. When the reciprocals of the initial reaction velocities were plotted against the reciprocals of the Mg^{2+} ion concentrations according to the method of Lineweaver & Burk (1934), straight lines were obtained. Fig. 3 shows the results obtained for the forward reaction at pH 7·2 and 7·9, and Fig. 4 shows the results obtained for the reverse reaction at pH 8·4 and 7·9. Similar results were obtained when the Mn^{2+} ion concentration in the reverse reaction system was varied (Fig. 4).

The above results conform to a Michaelis–Menten relation, i.e. to the equation $v = V_x/(K_x + x)$, where v is initial reaction velocity, V is maximum reaction velocity, x is cation concentration and K_x is the dissociation constant of the metal(Me)–APK complex formed according to the equation:

$$APK + Me^{2+} \rightleftharpoons Me\text{–}APK.$$

They are, therefore, consistent with the hypothesis that one Mg^{2+} or Mn^{2+} ion combines with each active centre of the enzyme to form the active complex.

The calculated dissociation constants and $V_{max.}$ values are listed in Table 1. When the concentrations of ADP and ATP are sufficiently high to saturate the enzyme, namely, 10^{-3} M and 5×10^{-3} M respectively, it will be seen that the dissociation constant of the Mg–APK complex at pH 7·9 differs as determined in the forward and reverse reactions. Moreover, the dissociation constant of the Mg–APK complex, as determined in the forward reaction, was increased as a result of a fivefold increase of the ADP concentration. The dissociation constant of the metal–APK complex is the same for both Mg^{2+} and Mn^{2+} ions, although the $V_{max.}$ value is greater in the presence of Mn^{2+} ions. This finding is in agreement with the previous finding (Morrison *et al.* 1957) that the activation of APK by Mn^{2+} ions is greater than by Mg^{2+} ions.

Table 1. *Dissociation constants of* Mg–*APK and* Mn–*APK complexes*

Conditions for the forward reaction were as described in Fig. 3; those for the reverse reaction as described in Fig. 4.

Metal	pH	Concn. of ADP (mM)	Dissociation constant (mM)	$V_{max.}$ (μmoles/mg./min.)
		Forward reaction		
Mg^{2+}	7·2	1	0·54	57·0
	7·9	1	0·43	45·5
	7·9	5	1·0	—
		Reverse reaction ([ATP] = 5 mM)		
Mg^{2+}	7·9	—	0·87	7·5
	8·4	—	1·5	10·7
Mn^{2+}	8·4	—	1·5	12·5

Effect of substrate concentrations on the reaction velocity. (*a*) Forward reaction. The effect of ADP on the reaction velocity of the forward reaction at pH 7·2 was determined under two sets of experimental conditions. In the first, all the components of the reaction system, including Mg^{2+} ions, were held constant and the ADP concentration was varied. The results plotted according to the method of Lineweaver & Burk (1934) are shown in Fig. 5. With a Mg^{2+} ion concentration of 5×10^{-3} M, a Michaelis–Menten relation is obtained over a limited range of ADP concentrations, i.e. between 10^{-3} and 4×10^{-4} M. Within this range, the K_m value of APK for ADP was calculated to be $2{\cdot}4 \times 10^{-4}$ M. As would be expected from the results shown in Fig. 2, when the ADP concentration falls below 4×10^{-4} M in the presence of 5×10^{-3} M-Mg^{2+} ions the reaction velocity decreases more markedly, so that the reciprocal values are higher than those required for a straight-line plot. Thus at the lower concentration of ADP, the Michaelis–Menten relation does not hold. When the Mg^{2+} ion concentration was varied simultaneously with the ADP concentration so as to maintain a Mg^{2+} ion : ADP ratio of 5 : 1, a Michaelis–Menten relation was obtained over the whole range of ADP concentrations investigated (Fig. 5). Under these conditions, the K_m value was calculated to be 3×10^{-4} M. This value is similar to that obtained above.

The effect of PA concentration on the reaction velocity in the presence of Mg^{2+} ions at a concentration of 5×10^{-3} M is also shown in Fig. 5. It was

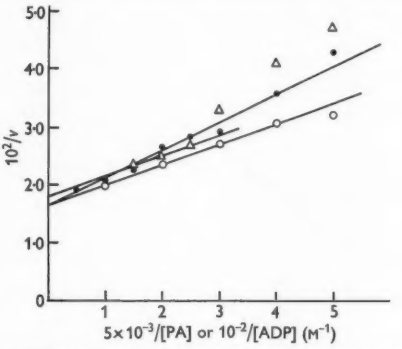

Fig. 5. Effect of PA and ADP concentrations on the initial velocity of the forward reaction. The results are plotted as described by Lineweaver & Burk (1934). The reaction mixture in all cases contained *N*-ethylmorpholine (0·06 M, pH 7·2) and APK (1 μg. of protein/ml.). ○, Variable substrate: PA, Mg^{2+} ions (5 mM), ADP (2 mM); △, variable substrate: ADP, Mg^{2+} ions (5 mM), PA (10^{-2} M); ●, variable substrate: ADP (Mg^{2+} ions also varied to give Mg : ADP = 5 : 1). Total volume 1·0 ml.; temp. 5°. v = μmoles/mg./min.

calculated that the K_m value of APK for PA was $1{\cdot}2 \times 10^{-3}$ M. The same value was obtained when the Mg^{2+} ion concentration was increased to 10^{-2} M. It will be noted that identical maximum velocities were obtained irrespective of whether ADP or PA was the variable substrate. Thus in each case the concentrations of the non-variable substrates were sufficient to saturate the enzyme.

(*b*) Reverse reaction. The relationship between the initial reaction velocity of the reverse reaction and the ATP concentration was determined under conditions where the Mg^{2+} ion concentration was fixed at 10^{-2} M and also where the ratio of Mg^{2+} ion:ATP was maintained at 1:1. The results plotted according to the method of Lineweaver & Burk (1934) are shown in Fig. 6. In the latter case the reaction velocity at each level of ATP was lower than that obtained when the Mg^{2+} ion concentration was fixed at 10^{-2} M except for the one instance where the Mg^{2+} ion:ATP ratio of 1:1 is common to each plot.

It can be seen (Fig. 6) that when the arginine concentration was varied and the other components of the system were held constant a straight line was obtained when the results were plotted as described by Lineweaver & Burk (1934). It will be noted that the maximum velocity was greater than that obtained with ATP as the variable substrate. It would seem therefore that the enzyme was not saturated with respect to arginine under the conditions chosen for the determination of the K_m of APK for ATP. The K_m of APK for arginine was calculated to be $3{\cdot}2 \times 10^{-3}$ M.

A summary of the K_m and $V_{max.}$ values obtained for the substrates of APK is given in Table 2.

Inhibition of the forward and reverse reactions by the reaction products. It was of interest to determine whether or not the products of the forward and the reverse reactions had any effect on the velocity of the reaction. For technical reasons it was not possible to test the effect of arginine in the forward reaction or PA in the reverse reaction. However, it was possible to test the effect of ATP in the forward reaction and ADP in the reverse reaction. Table 3 shows that ATP does not inhibit the forward reaction when added at the same concentration as the ADP, and that it is only when the ATP concentration is 10 times that of the ADP that inhibition occurs. There was only a low degree of inhibition by adenylic acid. These results suggest that the affinity of APK for ADP under the conditions of the forward reaction is very much greater than for ATP or adenylic acid. Alternatively, the results could be explained as being due to a reduction of the Mg^{2+} ion concentration and hence the concentration of the active Mg–APK complex as a result of the formation of Mg–ATP and Mg–adenylic acid complexes.

In contrast to the above results there is marked inhibition of the reverse reaction by ADP. Fig. 7 shows that the inhibition is competitive, the degree of inhibition depending both on the concentration of

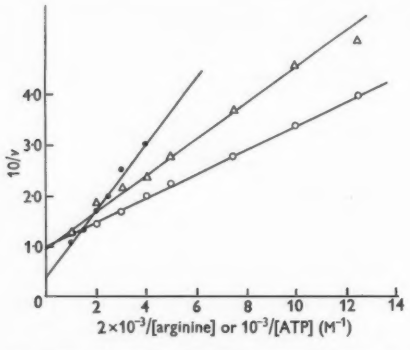

Fig. 6. Effect of arginine and ATP concentrations on the initial velocity of the reverse reaction. The results are plotted as described by Lineweaver & Burk (1934). The reaction mixture in all cases contained *N*-ethylmorpholine (0·06 M, pH 8·4) and APK (15 μg. of protein/ml.). ●, Variable substrate: arginine, Mg^{2+} ions (10^{-2} M), ATP (10^{-2} M); ○, variable substrate: ATP, Mg (10^{-2} M), arginine (10^{-2} M); △, variable substrate: ATP (Mg^{2+} ions also varied to give Mg:ATP = 1:1). Total volume 1·0 ml.; temp. 5°. v = μmoles/mg./min.

Table 2. *K_m values and maximum velocities*

Conditions were as described in Figs. 5 and 6.

Substrate	K_m (mM)	$V_{max.}$ (μmoles/mg./min.)	
	Forward reaction (pH 7·2)		
PA	1·2	61·0	—
ADP	2·4	61·0	Mg^{2+} ions constant
	3·0	56·0	Mg^{2+} ions: ADP = 5:1
	Reverse reaction (pH 8·4)		
Arginine	3·2	25·0	—
ATP	2·5	10·5	Mg^{2+} ions constant
	3·8	9·8	Mg^{2+} ions: ATP = 1:1

Table 3. *Inhibition of the forward reaction by ATP and AMP*

The reaction mixture contained PA (5×10^{-3} M), ADP (10^{-3} M), Mg^{2+} ions (10^{-2} M), *N*-ethylmorpholine (0·06 M, pH 7·2) and APK (1 μg. of protein/ml.). Total volume, 1·0 ml. Temp. 5°.

Addition	Concn. (mM)	Inhibition (%)
ATP	10	54
	1	0
Adenylic acid	4	26
	1	16

the ADP and ATP. Calculation of the K_I value for ADP gave a figure of $1{\cdot}5 \times 10^{-3}$ M, which is the same as that obtained for the dissociation constant of the APK–ATP complex.

Effect of temperature on the reaction velocity. The effect of temperature on the reaction velocity is shown in Fig. 8. Two Arrhenius plots are shown; one for the forward reaction at pH 7·2 and the other for the reverse reaction at pH 8·4. In order to measure true initial reaction velocities, the same enzyme concentration was used at all temperatures, but the reaction time was progressively decreased with increasing temperature. The forward reaction followed the Arrhenius equation over the range 0–35°. From the slope of the line, the apparent activation energy was calculated to be 9500 cal./mole. The Arrhenius equation held also for the reverse reaction over the same temperature range, although there was a sharp break at about 10°. The apparent activation energy was 21 500 below 10° and 9800 cal./mole above 10°.

Equilibrium studies. The effect of pH and Mg^{2+} ion concentration on the percentage phosphorylation of arginine is shown in Fig. 9. It is apparent that increasing pH favours the phosphorylation of arginine at the three concentrations of Mg^{2+} ions used and that the degree of phosphorylation is dependent on the Mg^{2+} ion concentration at any particular pH. As the Mg^{2+} ion concentration decreases the percentage phosphorylation of arginine increases.

Enzymic activity in the absence of added Mg^{2+} *ions.* A comparison was made of the K_m values of APK for ADP and PA in the presence and absence of Mg^{2+} ions, and for this purpose an APK preparation before dilution with glycerol was dialysed against 0·01 M phosphate buffer (pH 7·4). In the absence of Mg^{2+} ions the time–activity curve (with a protein concentration of 5 μg./ml.) showed a rapid fall off of the reaction rate. This occurred after 4 min. when only 0·13 μmole of arginine had been released in the presence of 5 μmoles of PA and 1 μmole of ADP; up to 4 min. the reaction was linear. Thus for the determination of the K_m values the volume of the reaction mixture was doubled and the reaction time reduced to 3 min.

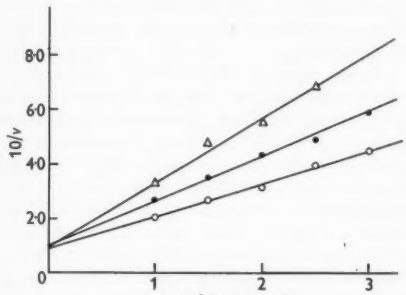

Fig. 7. Competitive inhibition of the reverse reaction by ADP with ATP as the variable substrate. The results are plotted according to the method of Lineweaver & Burk (1934). The reaction mixture contained *N*-ethylmorpholine (0·06 M, pH 8·4), arginine (10^{-2} M), Mg^{2+} ions (10^{-2} M) and APK (15 μg. of protein/ml.). Total volume, 1·0 ml.; temp., 5°. ○, No added ADP; ●, ADP (mM); △, ADP (2 mM). v = μmoles/mg./min.

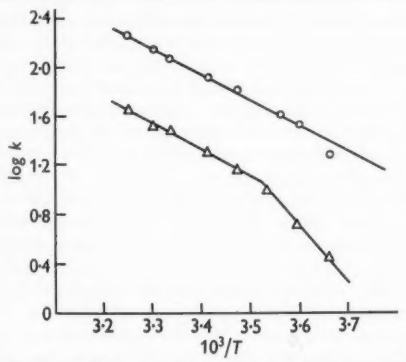

Fig. 8. Effect of temperature on the initial velocity of the forward and reverse reaction. The results are graphed as Arrhenius plots. Conditions for the forward reaction were as described in Fig. 2; conditions for the reverse reaction were as described in Fig. 1. T = absolute temp.; k = μmoles/mg./min. ○, Forward reaction; △, reverse reaction.

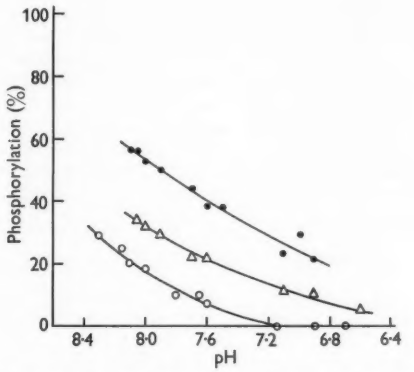

Fig. 9. Effect of pH and Mg^{2+} ion concentration on the percentage phosphorylation of arginine. The reaction mixture contained PA (5 mM), ADP (mM), *N*-ethylmorpholine (0·06 M) and APK (46 μg. of protein/ml.); incubated for 2 hr. at 5°. The pH was checked by means of a glass electrode in a duplicate set of tubes. ○, Mg^{2+} ions (10^{-2} M); △, Mg^{2+} ions (mM); ●, Mg^{2+} ions (2×10^{-4} M).

It was found that the K_m of APK for ADP was $1{\cdot}5 \times 10^{-4}$M as compared with $3{\cdot}0 \times 10^{-4}$M in the presence of Mg^{2+} ions, whereas the K_m of APK for PA was $2{\cdot}2 \times 10^{-3}$M as compared with $1{\cdot}2 \times 10^{-3}$M in the presence of Mg^{2+} ions.

The dialysed APK preparation also catalysed the reverse reaction in the absence of Mg^{2+} ions, but the amount of protein required to obtain suitable reaction rates was so high that it interfered with the P estimation. Hence it was not possible to determine the K_m values of APK for ATP and arginine.

Inhibition of the forward reaction by Ca^{2+} *ions.* When Ca^{2+} ions were added to the forward reaction catalysed by APK in the absence of Mg^{2+} ions, there was no inhibition up to concentrations of 10^{-2}M. On the other hand, in the presence of 10^{-2}M-Mg^{2+} ions, 10^{-2}M-Ca^{2+} ions produced an inhibition of 72%. Thus the effect of Ca^{2+} ions is to lower the increase in activity due to Mg^{2+} ions. Further investigation of the inhibition of the reaction by Ca^{2+} ions showed that it was competitive (Fig. 10), the degree of inhibition depending upon the relative concentrations of Mg^{2+} and Ca^{2+} ions. It was calculated that the K_I value for Ca^{2+} ions was $8{\cdot}3 \times 10^{-5}$M as compared with a dissociation constant of $3{\cdot}6 \times 10^{-4}$M for Mg–APK. The affinity of APK for Ca^{2+} ions is therefore much greater than its affinity for Mg^{2+} ions.

DISCUSSION

The nature of the activation produced by Mg^{2+} ions in transphosphorylation reactions is of some interest; on general grounds it is possible that a Mg–ATP complex is formed which acts as the 'active' substrate. Such a conclusion has been reached by Kuby, Noda & Lardy (1954), Hers (1952) and Liébecq (1953) as a result of the study of reactions catalysed by creatine phosphokinase, fructokinase and hexokinase respectively. They found that maximum reaction velocities were attained when the Mg^{2+} ion and ATP concentrations were approximately equal. A similar result has also been obtained in the present work but seems open to other interpretations, for Mg^{2+} ions could also act by influencing the velocity of the breakdown of the enzyme–substrate complex, or by reacting with the apoenzyme to form an active Mg–enzyme complex.

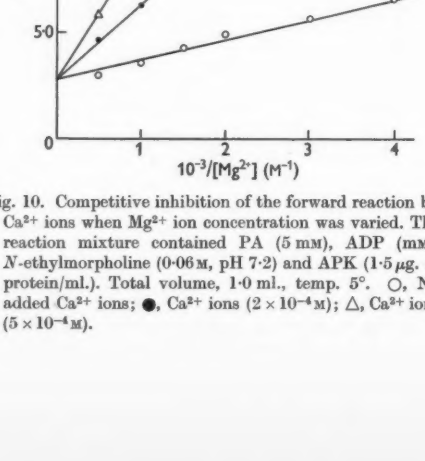

Fig. 10. Competitive inhibition of the forward reaction by Ca^{2+} ions when Mg^{2+} ion concentration was varied. The reaction mixture contained PA (5 mM), ADP (mM), *N*-ethylmorpholine (0·06M, pH 7·2) and APK (1·5 μg. of protein/ml.). Total volume, 1·0 ml., temp. 5°. ○, No added Ca^{2+} ions; ●, Ca^{2+} ions (2×10^{-4}M); △, Ca^{2+} ions (5×10^{-4}M).

In assessing the validity of the conclusion drawn by the above workers it should be remembered that it is based upon a relationship obtained when enzymic activities, at varying concentrations of ATP, are plotted against the concentration of added Mg^{2+} ions, and that this is tacitly assumed to be equal to the concentration of free Mg^{2+} ions. Such an assumption is, however, invalid, for as Burton & Krebs (1953) have shown, both ATP and ADP form complexes with Mg^{2+} ions, so that the concentration of 'free Mg^{2+} ions', 'free ATP' and Mg–ATP will vary according to the amounts of Mg^{2+} ions and ATP added.

The relationship between the velocity of the APK reaction (in both the forward and reverse directions) and the Mg^{2+} ion concentration is consistent with the idea that Mg^{2+} ions react with the enzyme in the ratio of one Mg^{2+} ion to each active centre, according to the equation

$$Mg^{2+} + APK \rightleftharpoons Mg\text{–}APK.$$

Thus the Mg–APK complex can be considered as the active-enzyme complex. The dissociation constant of Mg–APK as determined from the plot of $1/v$ against $1/Mg^{2+}$ ions (where the concentration of Mg^{2+} ions is taken to be the same as that added) will not be a true dissociation constant for the Mg–APK complex. The value so calculated will lie between the true dissociation constant of the Mg–APK complex and that of either Mg–ATP or Mg–ADP, and will depend on the relative dissociation constants of Mg–APK and Mg–ATP or Mg–ADP. It would be expected that, under the same experimental conditions and provided that there are no secondary reactions between Mg^{2+} ions and the substrates, the dissociation constant of the Mg–enzyme complex would be the same irrespective of whether it was determined as a result of study of the forward or reverse reactions. This expectation is not realized experimentally, for the results (Table 1) show that at pH 7·9 the value is lower when calculated from the forward reaction than when calculated from the reverse. Thus less Mg^{2+} ions are apparently required to half-saturate

the enzyme in the presence of ADP than in the presence of ATP. Such a result is, however, consistent with the interpretation that Mg^{2+} ions react with the enzyme, for the dissociation constant of Mg–ADP is greater than that of Mg–ATP and thus more Mg^{2+} ions would be available to interact with the enzyme. It can also be seen (Table 1) that a fivefold increase in ADP concentration results in an increase in the dissociation constant of Mg–APK, which is consistent with the idea that ADP reduces the amount of Mg^{2+} ions available for reaction with the enzyme.

The differences in the values for the dissociation constant may be accounted for by the fact that the abscissae values of the plots (Figs. 3, 4) represent the concentrations of Mg^{2+} ions added and thus do not allow for any reduction as a result of interaction with ADP or ATP. It is, however, possible to calculate the 'free Mg^{2+} ion' concentration from the dissociation constants of Mg–ATP and Mg–ADP (Burton & Krebs, 1953), and for the purpose of calculation it has been assumed that Mg^{2+} ions react, in the systems investigated, only with ATP, ADP and APK; all other possible reactions have been neglected. On this basis the Mg^{2+} ions which are not in combination with ATP or ADP are referred to as 'free Mg^{2+} ions'. If the reciprocals of the values so obtained are plotted against $1/v$ the dissociation constants of Mg–APK as determined under various conditions may be calculated and are shown in bold type in Table 4. It will be seen that these values are very close to each other and differ much less than those calculated directly from Figs. 3 and 4. They are also considerably lower than these latter, which are dependent upon the concentration of ATP or ADP in the system and the dissociation constants of Mg–ATP and Mg–ADP. These results indicate that the role of Mg^{2+} ions in the APK reaction lies in a combination of the metal with the enzyme and that the formation of Mg–ADP and Mg–ATP are inevitable side reactions.

A characteristic feature of the results plotted in Fig. 1 is that maximum enzymic activities are obtained at varying concentrations of Mg^{2+} ions, depending on the concentrations of ATP. If, however, the activities are replotted against the concentrations of 'free Mg^{2+} ions' determined as before, it can be shown that maximum enzymic activity is obtained when the latter is 2×10^{-3} M and that this value is independent of the concentration of ATP. It is also worthy of note that no relationship between enzymic activity and Mg–ATP concentration could be demonstrated. In the forward reaction (Fig. 2) maximum enzymic activity is again reached at Mg^{2+} ion concentrations which are dependent upon the ADP concentration. There is, however, no simple relationship between the concentrations of ADP and Mg^{2+} ions when maximum enzymic activity is reached. If these results are recalculated and the enzymic activity is plotted against 'free Mg^{2+} ion' concentration, it is found that maximum enzymic activity is reached at each level of ADP when the concentration of 'free Mg^{2+} ions' is $2{\cdot}5 \times 10^{-3}$ M. This value is similar to that obtained for the reverse reaction.

It will be noted that the maximum velocities in the forward reaction (Fig. 2) fall within a much narrower range of Mg^{2+} ion concentrations, and that they are much closer to the true values than is the case with the reverse reaction (Fig. 1). These findings are due to the fact that the amounts of ADP added in the forward reaction were less than the amounts of ATP added for the reverse reaction, and since the dissociation constant of Mg–ADP is higher than that of Mg–ATP it follows that the values for concentrations of 'free Mg^{2+} ions' in the forward reaction must be closer to those for total Mg^{2+} ions than is the case in the reverse reaction.

The decrease in velocity of the forward reaction (Fig. 2) in the presence of high concentrations of Mg^{2+} ions is due to a reduction of 'free ADP' concentrations, as a result of interaction with Mg^{2+} ions. In this case the ratio of Mg^{2+} ions:ADP was much greater than was the ratio of Mg^{2+} ions:ATP in the reverse reaction, where no fall off of reaction velocity was noted (Fig. 1).

When the K_m of APK for ADP was determined in the presence of a constant amount of Mg^{2+} ions

Table 4. *Dissociation constants of* Mg–*APK from plots of* $1/v$ *against* 1/*total* Mg *and* 1/'*free* Mg^{2+} *ions*'

Determined in the presence of	Concn. of ADP or ATP (mM)	Calculated dissociation constant		
		pH 7·2	pH 7·9	pH 8·4
ADP	1	$5{\cdot}4 \times 10^{-4}$*	$4{\cdot}3 \times 10^{-4}$	—
		$3{\cdot}6 \times 10^{-4}$†	**$3{\cdot}1 \times 10^{-4}$**	—
	5	—	$1{\cdot}0 \times 10^{-3}$	—
			$3{\cdot}3 \times 10^{-4}$	—
ATP	5	—	$8{\cdot}7 \times 10^{-4}$	$1{\cdot}5 \times 10^{-3}$
			$2{\cdot}4 \times 10^{-4}$	**$4{\cdot}7 \times 10^{-4}$**

* Calculated from a plot of $1/v$ against 1/total Mg^{2+} ions.
† Calculated from a plot of $1/v$ against 1/'free Mg^{2+} ions'.

a Michaelis–Menten relation was obtained only over a limited range of ADP concentrations, for the velocity was much less at lower levels of ADP than that required. This was due to a reduction of the ADP concentrations by Mg^{2+} ions, for a Michaelis–Menten relation was obtained over the entire range of ADP concentrations when $1/v$ was plotted against 1/'free ADP' rather than 1/total ADP. The K_m value obtained from this plot (Table 5) must be regarded as the true value and it will be noted that it is appreciably lower than that calculated on the basis of total ADP. When the Mg^{2+} ion concentration was varied simultaneously with the ADP to maintain a Mg:ADP ratio of 5:1 a Michaelis–Menten relation was obtained over the range of ADP concentrations studied. The K_m value (Table 5) was close to that obtained when the Mg^{2+} ion concentration was kept constant, both values being calculated from a plot of $1/v$ against 1/total ADP. However, the K_m value calculated from a plot of $1/v$ against 1/'free ADP' is considerably higher than what is considered as the true value, and higher than that obtained on the basis of total ADP. This high value is probably due to the fact that as the Mg^{2+} ion concentration was reduced concomitantly with the ADP concentration (maintaining a Mg:ADP ratio = 5:1) it limited the concentration of the Mg–APK complex. Thus the fall off in rate was due to a reduction of both the ADP and Mg–APK concentrations.

When the ATP concentration was varied in the presence of a fixed concentration of Mg^{2+} ions and when both the ATP and Mg^{2+} ion concentrations were varied so as to maintain a Mg:ATP ratio of 1:1, a Michaelis–Menten relation between the reaction velocity and the ATP concentration was found (Fig. 6). It can be seen (Table 5) that although the K_m values calculated from a plot of $1/v$ against 1/total ATP differ slightly, they are considerably different from those calculated from a plot of $1/v$ against 1/'free ATP'. The value obtained from the latter plot in the presence of a fixed amount of Mg^{2+} ions must be considered as the true K_m value of APK for ATP. The high value calculated from a similar plot of the results obtained when the Mg^{2+} ion concentration was varied is probably due to the concentration of Mg^{2+} ions limiting the concentration of Mg–APK as was the case with the forward reaction.

Table 5. *K_m values of APK for ADP and ATP*

Substrate	Concn. of Mg^{2+} ions (M)	K_m value (M) (*a*)*	(*b*)†
ADP	5×10^{-3}	$2{\cdot}4 \times 10^{-4}$	$4{\cdot}3 \times 10^{-5}$
	Mg:ADP = 5:1	$3{\cdot}0 \times 10^{-4}$	$6{\cdot}6 \times 10^{-4}$
ATP	1×10^{-2}	$2{\cdot}5 \times 10^{-3}$	$2{\cdot}8 \times 10^{-4}$
	Mg:ATP = 1:1	$3{\cdot}8 \times 10^{-3}$	$6{\cdot}3 \times 10^{-3}$

* (*a*) Calculated from a plot of $1/v$ against 1/total substrate.

† (*b*) Calculated from a plot of $1/v$ against 1/'free substrate'.

The influence of Mg^{2+} ions on the reaction can also be seen from the equilibrium studies. As the concentration of Mg^{2+} ions is increased, the equilibrium shifts in favour of arginine and ATP. This can be explained as being due to the greater affinity of Mg^{2+} ions for ATP than for ADP. If Mg–ATP were the true substrate for the reaction, it would be expected that the equilibrium would be displaced in favour of PA and ADP as the concentration of Mg^{2+} ions, and thus of Mg–ATP, was increased.

There is additional supporting evidence to suggest that the function of Mg^{2+} ions is to form a complex with the apoenzyme. In the experiments carried out with the dialysed APK preparation to which no Mg^{2+} ions were added the enzyme showed activity in both the forward and reverse reactions. It was found that the K_m of APK for ADP was lower than when Mg^{2+} ions were present although higher than that calculated on the basis of the 'free ADP' present when Mg^{2+} ions were added; on the other hand, the K_m value of APK for PA was increased. Although no explanation can be offered for the higher value, the results do indicate that the binding of Mg^{2+} ions by PA must be sufficiently weak so as to have no effect on the reaction. The activity of the enzyme in the absence of added Mg^{2+} ions is probably due to Mg^{2+} ions bound to the enzyme. Spectrographic analysis (Morrison *et al.* 1957) has shown that Mg^{2+} ions are present in a dialysed APK preparation.

Further evidence for the idea that Mg^{2+} ions react with APK to form an active Mg–APK complex comes from the results of the inhibition of APK by Ca^{2+} ions. Ca^{2+} ions have no effect on the forward reaction catalysed by APK in the absence of Mg^{2+} ions, whereas they competitively inhibit the enzyme in the presence of Mg^{2+} ions. Thus the interaction of Ca^{2+} ions and ADP to form Ca–ADP does not appreciably reduce the concentration of free ADP. This can be explained by the fact that the K_m value of APK for ADP ($4{\cdot}3 \times 10^{-5}$ M) is much lower than the dissociation constant of Ca–ADP ($1{\cdot}8 \times 10^{-4}$ M; Distefano & Neuman, 1953). The inhibition of the reaction when Mg^{2+} ions are present must therefore be due to the competition by Ca^{2+} and Mg^{2+} ions for the same site on the enzyme surface. It is noteworthy that the affinity of APK for Ca^{2+} ions is greater than for Mg^{2+} ions, although there is no activation by Ca^{2+} ions. These results also imply that in APK as isolated, the linkage of Mg^{2+} ions to the enzyme is different from and stronger than that resulting from the addition of Mg^{2+} ions to the enzyme system.

The competitive inhibition of the reverse reaction by ADP and the inhibition of the forward reaction by ATP suggest that ATP and ADP are bound to APK at the same site on the enzyme surface. It is possible that this linkage is through Mg^{2+} ions, as this metal complexes predominantly in *O*-type linkages (Williams, 1953). The fact that ATP cannot be replaced by inosine triphosphate in the reaction (Morrison *et al.* 1957) indicates that the adenine moiety of ATP is also involved in the linkage of ATP or ADP to the enzyme.

It is believed that the present results indicate that Mg^{2+} ions react with the apoenzyme to form an active Mg–APK complex, and that reaction of free ADP or ATP with the active enzyme involves linkage with Mg^{2+} ions. It is possible that PA and arginine are attached at another site on the enzyme surface in sufficiently close proximity to the ADP–ATP site so that P can be directly transferred without liberation as inorganic P. This hypothesis, inasmuch as it concerns the reaction investigated, may be schematically represented as in Fig. 11.

Although this scheme is based on the results of experiments in which Mg^{2+} ions were used to activate the enzyme, it should be borne in mind that APK is also activated by Mn^{2+} ions. It is, therefore, probable that the mechanism of the activation of APK by Mn^{2+} ions is similar to that postulated for Mg^{2+} ions.

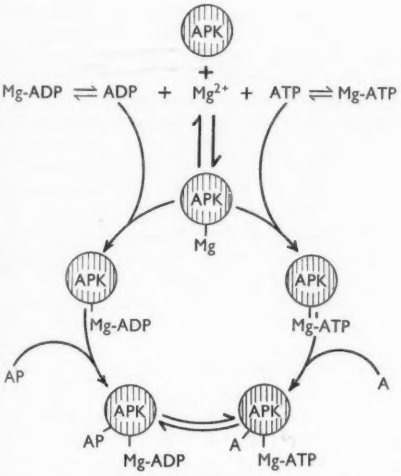

Fig. 11. Schematic representation of the mechanism of the transphosphorylation reaction catalysed by APK. All reactions are reversible. The abbreviations AP and A represent phosphoarginine and arginine respectively.

It should be pointed out that recalculation of the results of Kuby *et al.* (1954), Hers (1952) and Liébecq (1953) might well indicate that a similar central reaction, i.e. formation of a metal–enzyme complex, is concerned in the reactions catalysed by creatine phosphokinase, fructokinase and hexokinase, and that this type of reaction may be one generally applicable to phosphate-transfer reactions.

SUMMARY

1. The kinetics of the reaction, phosphoarginine + adenosine diphosphate ⇌ arginine + adenosine triphosphate, catalysed by arginine phosphokinase have been studied.

2. It has been shown that when enzymic activity is plotted against the total Mg^{2+} ion concentration, maximum activity is reached at a concentration of Mg^{2+} ions which is dependent on the concentration of adenine diphosphate (ADP) or adenosine triphosphate (ATP). When enzymic activity is plotted against the concentration of 'free Mg^{2+} ions', maximum activity is reached at a Mg^{2+} ion concentration which is independent of the concentration of ADP or ATP.

3. A Michaelis–Menten relation between enzymic activity and the concentration of Mg^{2+} and Mn^{2+} ions was demonstrated. The dissociation constants of the magnesium–arginine phosphokinase complex, determined under various conditions, were calculated from plots of $1/v$ against 1/total Mg^{2+} ions and $1/v$ against 1/'free Mg^{2+} ions'.

4. The K_m values of the four substrates of the reaction were determined. Those for ADP and ATP were calculated from plots of $1/v$ against 1/total substrate and $1/v$ against 1/'free substrate'.

5. The forward reaction was inhibited by ATP and adenylic acid; the reverse reaction was inhibited competitively by ADP. It was concluded that ADP and ATP are bound to the enzyme at the same site.

6. There was competitive inhibition of the forward reaction by Ca^{2+} ions.

7. The effect of temperature on the velocity of the forward and reverse reactions was studied and the apparent activation energies were calculated.

8. The equilibrium of the reaction was influenced by pH and the concentration of Mg^{2+} ions.

9. The conclusion was reached that Mg^{2+} ions react with arginine phosphokinase to form an active magnesium–arginine phosphokinase complex which in turn reacts with the free forms of ATP and ADP.

It is a pleasure to express our thanks to Mr D. Maguire for skilled technical assistance.

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Studies in the Biochemistry of Micro-organisms

99. METABOLIC PRODUCTS OF *ASPERGILLUS VERSICOLOR* (VUILLEMIN) TIRABOSCHI*

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The first examination in this Laboratory of the metabolic products obtained from the mycelium of *Aspergillus versicolor* (Vuillemin) Tiraboschi was undertaken by Abou-Zeid (1953). He isolated in very small yield two new coloured crystalline metabolites, *A*, m.p. 240–241°, and *B*, m.p. 233–234°, in addition to mannitol. The amounts of *A* and *B* obtained were insufficient for complete characterization but a few properties and colour reactions were noted.

One of us (Hammady, 1954), using a later isolation of the same organism, obtained the same three products. Product *A*, formed in relatively good yield, was obtained as pale-yellow needles of m.p. 247°, and was allocated the molecular formula $C_{18}H_{12}O_6$. The general properties, derivatives and some breakdown products were described.

Davies, Roberts & Wallwork (1956) recently announced that early in 1955 they had commenced an investigation of *A. versicolor* and had obtained from the dried mycelium of one strain a metabolite crystallizing in pale-yellow needles of m.p. 243°. They drew attention to the work of Hatsuda & Kuyama (1954) and of Hatsuda, Kuyama & Terashima (1954), who had isolated the same metabolite and named it sterigmatocystin. Hatsuda *et al.* (1954) ascribed to sterigmatocystin the molecular formula $C_{15}H_{12}O_5$ and allocated to it a structural formula. Davies *et al.* (1956), adopting the name sterigmatocystin, determined the molecular weight by the Rast method and by the X-ray crystallographic method. From these results and the combustion figures they concluded that the molecular formula for sterigmatocystin is $C_{18}H_{14}O_6$, both empirical and structural formulae given by Hatsuda *et al.* (1954) being thus incorrect.

It was then evident that product *A* isolated in this Laboratory was the same as sterigmatocystin. Through the courtesy of Dr Roberts we were able to compare our product directly with a specimen isolated by the Nottingham School; a mixed-melting-point determination showed they were identical. Since the work carried out on this subject in this School has been reported only in Ph.D. theses of London University it is appropriate to record briefly for wider circulation the results therein embodied which relate to the metabolic products of *A. versicolor*.

Some strains of the fungus when grown on Czapek–Dox medium produced a coloured mycelium, which, after drying, was extracted with solvents. Light petroleum afforded an orange-coloured solid from which product *A* (sterigmatocystin) was obtained. On subsequent ether extraction of the mycelium product *B* was isolated. It formed orange needles of m.p. 233–234°. Further extraction of the mycelium with methanol afforded mannitol.

Sterigmatocystin is optically active and contains one methoxyl group. The following derivatives have been prepared: diacetyl, monomethoxy, monoethoxy compounds and a brominated derivative. When refluxed with 25% ethanolic potassium hydroxide, sterigmatocystin furnished an optically inactive isomer which afforded a monomethyl ether and a diacetate. This product, which we term *iso*-sterigmatocystin, shows an ultraviolet-absorption curve very similar to that of sterigmatocystin, indicating that the conjugated system giving rise to the absorption is still present. Some change other than mere racemization must have occurred,

* Part 98: Neelakantan, Pocker & Raistrick (1956).

however, since *iso*sterigmatocystin is readily soluble in aqueous sodium hydroxide whereas sterigmatocystin is insoluble in this reagent. On distillation of sterigmatocystin with zinc dust a small amount of crystalline material was isolated, which from its ultraviolet-absorption spectrum appeared to be anthracene or a homologue thereof. On heating sterigmatocystin with fused potassium hydroxide resorcinol was obtained together with acetic acid. The combustion figures for the diacetate of sterigmatocystin indicate that it has the empirical formula $C_{22}H_{18}O_9$, and thus contains a molecule of water in excess of the formula for a simple diacetate; this may, of course, be water of crystallization. *iso*Sterigmatocystin forms the normal diacetate $C_{22}H_{16}O_8$. No evidence of the presence of a reactive carbonyl group in sterigmatocystin could be obtained. The figures obtained for methoxyl determinations on sterigmatocystin and its derivatives were consistently too high; this appears to be a characteristic of the substance.

Sterigmatocystin contains six oxygen atoms. One of these is present as a methoxyl group, two further oxygen atoms are present as hydroxyl groups, one of which can be methylated and both of which can be acetylated. This leaves a further three oxygens whose function is not as yet ascertained. Little can be said of the carbon skeleton in the light of this work, although the production of resorcinol by potassium hydroxide fusion and of anthracene by zinc-dust distillation suggest the presence of one or more aromatic rings. There may be present an aliphatic link containing the asymmetric carbon atom which confers optical activity. The hydroxyl group which resists methylation but not acetylation could well be part of this structure. Further work to elucidate the structure is by mutual agreement to be undertaken by the Nottingham School, who inform us that they are now in accord with our molecular formula of $C_{18}H_{12}O_6$ for sterigmatocystin (personal communication from Dr J. C. Roberts).

For product *B*, the evidence obtained is too scanty to permit any definite conclusions as to its nature. Whereas the colour reactions and the ultraviolet absorption agree well with a polyhydroxyanthraquinone structure, the hydrogen:carbon ratio as determined by combustion is too high to accord with this hypothesis. Product *B* does not appear to be identical with versicolorin (cf. Hatsuda & Kuyama, 1955; Hatsuda, Kuyama & Terashima, 1955) since it differs considerably in melting point and also shows optical activity.

EXPERIMENTAL

All melting points are uncorrected. Elementary analyses and acetyl determinations are by Drs Weiler and Strauss, Oxford, except where otherwise stated. Methoxyl determinations are by one of us (I. M. M. H.).

Organism

The culture originally used in this investigation (Abou-Zeid, 1953) was a strain (L.S.H.T.M. Cat. no. 247) of *Aspergillus versicolor* (Vuillemin) Tiraboschi received from the Timber Research Laboratories, South Africa (their Cat. no. 2543). This afforded only minimal yields of sterigmatocystin, but slightly larger amounts of product *B*. In the continuation of this work (Hammady, 1954) a strain (L.S.H.T.M. Cat. no. A 262) was employed which was isolated in this Laboratory from an Australian-soil sample by Mrs S. Marcus. When grown on Czapek–Dox glucose medium the fungus produced a mycelium which was convoluted and yellowish white on the upper surface with brown droplets, the reverse being brown to orange–brown in colour. On extraction it afforded sterigmatocystin in resonable yield, product *B* in minimal yield.

During the course of the investigation thirteen other strains of the organism were examined for production of the characteristic products. Of these, eight produced no pigment, four were inefficient pigment producers, but one was found to afford sterigmatocystin in better yield than strain A 262, although the yield of *B* was again minimal. With this strain (L.S.H.T.M. Cat. no. Ac 59), obtained from the Centraalbureau voor Schimmelcultures Baarn (ex Bainier), the mycelium after incubation for 21 days was orange to green in colour on the upper surface, with orange reverse. In the later work this strain Ac 59 was used in place of A 262 to prepare a stock of sterigmatocystin.

Method of culture

The usual Czapek–Dox medium of the following composition was employed: $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; glucose, 50 g.; water to 1 l. The medium was distributed (350 ml./flask) in 1 l. conical flasks plugged with cotton wool and sterilized by steaming on three successive days. Flasks were inoculated with a spore suspension from wort–agar slope cultures and incubated at 24° in the dark, usually for 21 days.

Harvesting and extraction of mycelium

The mycelium was separated from the culture solution by filtration, washed with water, pressed and dried in a vacuum at about 40°. It was then ground in a coffee mill and exhaustively extracted with light petroleum (b.p. 40–60°). The orange-coloured solid which separated consisted of crude sterigmatocystin (product *A*). It was repeatedly crystallized from amyl acetate, from which it separated as long pale-yellow needles, m.p. 247–248°. The yield was 40–50% of the crude material.

The mycelium was then extracted with ether. From the extract after concentration a dark brownish orange solid separated. This was crude product *B*. After several recrystallizations from $CHCl_3$ it was obtained as orange needles, m.p. 231–234°. The yield of pure material was only about 2·5% of the crude product.

Further extraction of the mycelium with methanol afforded brownish crystals, which by recrystallization were obtained as colourless needles, m.p. 166°. This product was identified as D-mannitol. The data obtained for four batches of strain A 262 and six batches of strain Ac 59 are shown in Table 1.

Table 1. *Culture characteristics and yields of crude products of strain A* 262 (*batches* 1–4) *and strain Ac* 59 (*batches* 5–10) *of* Aspergillus versicolor (*Vuillemin*) *Tiraboschi when grown on Czapek–Dox glucose medium at* 24°

Batch no.	No. of flasks	Period (days)	Residual glucose (%)	Wt. of dry mycelium (g.)	Crude sterigmatocystin		Crude product *B*	
					Wt. (g.)	M.p.	Wt. (g.)	M.p.
1	99	21	0·48	454	5·4	198–228°	2·6	180–200°
2	100	21	0·98	400	4·7	210–220	4·2	180–220
3	100	21	0·52	490	10·4	190–220	6·0	170–190
4	99	21	0·37	492	5·2	210–225	2·8	160–190
5	100	21	0·47	390	18·0	212–227	2·9	160–200
6	100	24	0·64	375	15·0	210–220	2·0	190–220
7	100	21	0·74	390	16·1	210–220	1·9	190–220
8	100	21	0·70	390	22·5	210–222	3·3	140–180
9	148	28	0·29	605	19·5	198–212	2·3	190–210
10	100	21	0·79	390	19·4	208–228	1·6	200–220

Properties of sterigmatocystin

When crystallized from amyl acetate sterigmatocystin forms long, pale-yellow needles of m.p. 247–248°. When the pure product was recrystallized from ethanol the m.p. dropped to 242–243°. The pure product sublimes unchanged *in vacuo* and then has m.p. 247°; $[\alpha]_{5461}^{20}$ −485°, $[\alpha]_{5790}^{20}$ −420°, $[\alpha]_{D}^{20}$ −398° in $CHCl_3$ (*c*, 1); light-absorption max., 250 and 326 mμ. in $CHCl_3$ (log ε 4·82 and 4·48 respectively). [Found: C, 65·8, 66·2, 66·7, 66·4; H, 4·0, 3·9, 4·0, 3·8; *C*-Me, nil; OMe (mean of several determinations) 10·8%. Mol.wt. (Rast) 344. Determination by Schoeller on a sublimed sample: C, 66·55, 66·5; H, 3·8, 3·8. $C_{18}H_{12}O_6$ requires C, 66·7; H, 3·7; one OMe group 9·6%. Mol.wt. 324.] It is sparingly soluble in most organic solvents in the cold, but is readily soluble in cold $CHCl_3$. It is insoluble in water and in aqueous NaOH. Its ethanolic solution gives a stable pale-green colour with ethanolic $FeCl_3$. It gives a green solution in conc. H_2SO_4 with very weak yellow fluorescence.

Derivatives of sterigmatocystin

Diacetyl sterigmatocystin. Sterigmatocystin (0·5 g.), anhydrous sodium acetate (1 g.) and acetic anhydride (5 ml.) were heated together in an oil bath at 140–150° for 6 hr. The mixture after cooling was poured into water with stirring and the solid obtained (0·4 g.) was collected and crystallized from pentanol or methanol giving the *diacetate* as colourless rods, m.p. 228–229°, $[\alpha]_{5461}^{22}$ −237° in ethanol (*c*, 3·8), which gave no colour with ethanolic $FeCl_3$. (Found: C, 61·9; 61·8; H, 4·3, 4·35; CH_2CO, 24·6, 24·8; OMe, 7·7. $C_{22}H_{16}O_8$,H_2O requires C, 61·95; H, 4·25; two CH_3CO groups, 20·2; one OMe group, 7·3%.)

Monomethyl ether of sterigmatocystin. Sterigmatocystin (0·5 g.) in dry acetone (50 ml.) was refluxed for 18 hr. with anhydrous K_2CO_3 (4 g.), and methyl iodide (7 g.) was added gradually at intervals. The acetone was distilled off and the residue treated with water. The insoluble material was crystallized from methanol, which afforded colourless needles of the *monomethyl ether*, 0·26 g., m.p. 271–272°; $[\alpha]_{5461}^{22}$ −459° in ethanol (*c*, 1). These gave no colour with ethanolic $FeCl_3$, and in conc. H_2SO_4 an emerald-green colour. (Found: C, 67·45; H, 4·5; OMe, 19·2, 19·9. $C_{19}H_{14}O_6$ requires C, 67·45; H, 4·2; two OMe groups, 18·3%.)

Monoethyl ether of sterigmatocystin. Sterigmatocystin (0·54 g.) in dry acetone (200 ml.) was refluxed with anhydrous K_2CO_3 (10 g.), and ethyl iodide (15 ml.) was added gradually during 18 hr. The product was treated with water after removal of acetone. The insoluble solid (0·48 g.) was crystallized from methanol, then from ethanol. The *monoethyl ether* separated as colourless needles, 0·2 g., m.p. 261–262°; $[\alpha]_{5461}^{22}$ −443° in ethanol (*c*, 1). (Found: C, 67·9, 67·8; H, 4·65, 4·7. $C_{20}H_{16}O_6$ requires C, 68·2; H, 4·6%.)

Bromination of sterigmatocystin

On the addition of saturated bromine water (15 ml.) to a solution of sterigmatocystin (0·24 g.) in dioxan (15 ml.) an immediate yellow ppt. formed. This was collected, washed with water and dried. The *product* (0·42 g.) crystallized from ethanol in yellow needles, m.p. 181–182° (decomp.), light-absorption max., 258 and 331 mμ. in ethanol ($E_{1\,cm.}^{1\%}$ 619 and 257 respectively). It was sparingly soluble in conc. aqueous NaOH. [Found: C, 36·5, 35·9; H, 2·0, 2·4; Br, 43·7, 44·0; OMe, 5·0, 5·0. Determination by I.C.I. Ltd., Welwyn: C, 35·8, 35·9; H, 2·4, 2·4; Br, 42·4, 42·35. The best fit, $C_{17}H_{13}O_7Br_3$, requires C, 35·9; H, 2·3; Br, 42·1; one OMe group, 5·45%. A more likely formula, $C_{18}H_{13}O_7Br_3$ (based on the addition of 4 Br and subsequent replacement of one Br by OH), requires C, 37·2; H, 2·3; Br, 41·3; OMe, 5·3%.]

Distillation of sterigmatocystin with zinc dust

An intimate mixture of sterigmatocystin (1 g.) and electrolytic-zinc dust (15 g.) was placed in a Pyrex tube (15 mm. diam., length of column 20 cm.) and sandwiched between two columns (10 cm. long) of zinc dust. The column was slowly heated (starting from the exit end of the tube) while a slow current of hydrogen was passed. A brown oil formed in the exit and gradually solidified. A fraction of this insoluble in light petroleum was sublimed (0·027 mg.). This had m.p. 195–196°. The u.v. absorption spectrum in ethanol closely resembled that of anthracene or β-methylanthracene but the $\lambda_{max.}$ agreed slightly better with those of anthracene. It is concluded that a product of zinc-dust distillation has the anthracene nucleus.

Fusion of sterigmatocystin with potassium hydroxide

Sterigmatocystin (2 g.), KOH (20 g.) and water (5 ml.) were stirred and heated slowly in a nickel crucible to 300–320°, at which temperature the melt was maintained for 20 min. The product, after cooling, was dissolved in water and extracted with ether, acidified and again extracted. The second ether extract was a brown gum, which slowly

began to crystallize. On sublimation a small amount of crystalline material was obtained. By further sublimations and recrystallization of the product from xylene and benzene colourless needles were eventually obtained of m.p. 110·5–111° (yield 0·2 g.) which gave a violet colour with aqueous $FeCl_3$. This was shown by the u.v. absorption spectrum and by a mixed m.p. determination with authentic material to be resorcinol. (Found: C, 65·5; H, 5·2. Calc. for $C_6H_6O_2$: C, 65·45; H, 5·5%.) The dibenzoate, m.p. 117–118°, showed no depression in m.p. when mixed with an authentic sample of dibenzoylresorcinol of the same m.p., prepared for comparison.

In a repetition of the KOH fusion the volatile acids liberated by acidification were distilled *in vacuo*. The neutralized distillate, concentrated to 3 ml., was treated with *p*-bromophenacyl bromide (1·2 g., the amount calculated from the titration) in 50% (v/v) aqueous ethanol (10 ml.) and the solution was refluxed for 2 hr. A crystalline product (1·06 g.) which separated after recrystallization from light petroleum formed colourless rods, m.p. 84–85°. The mixed m.p. with an authentic sample of *p*-bromophenacyl acetate, m.p. 84°, showed no depression.

Action of ethanolic potassium hydroxide on sterigmatocystin

Sterigmatocystin (0·35 g.) was refluxed with 25% ethanolic KOH (20 ml.). The substance dissolved completely in 6 hr. The heating was continued for a further 26 hr., and the solution became dark brown. After concentration at reduced pressure it deposited a light-brown solid which dissolved in water. Acidification to Congo red afforded a ppt. (0·2 g.) which after four crystallizations from *n*-butanol formed pale yellowish brown plates of *iso*sterigmatocystin, m.p. 234°; light-absorption max., 252 and 336 mμ. in ethanol (log ε 4·62 and 4·23 respectively). (Found: C, 66·7; H, 3·9; OMe, 11·1, 11·3. $C_{18}H_{12}O_6$ requires C, 66·7; H, 3·7; one OMe group, 9·6%.) Admixture with sterigmatocystin depressed the m.p. slightly (to 231°). The product was optically inactive and was readily soluble in 2N-NaOH, the solution being coloured yellow. It gave a green colour with ethanolic $FeCl_3$.

Diacetate of iso*sterigmatocystin*. This was prepared in similar manner to the diacetate of sterigmatocystin. From ethanol the *diacetate* was obtained in pale-brown needles, m.p. 197–198°. (Found: C, 64·25; H, 3·9; CH_3.CO, 22·2. $C_{22}H_{16}O_8$ requires C, 64·7; H, 3·9; two CH_3.CO groups, 21·1%.)

Methyl ether of iso*sterigmatocystin*. This was prepared in the same way as the corresponding product of sterigmatocystin and crystallized from a mixture of $CHCl_3$ and light petroleum; the *methyl ether* was obtained as colourless rods, m.p. 162–163°. (Found: C, 67·2; H, 4·6; OMe, 20·5. $C_{19}H_{14}O_6$ requires C, 67·45; H, 4·2; two OMe groups, 18·3%.)

Product B (*Abou-Zeid*, 1953)

The dry mycelium (400–500 g.) obtained from cultures of a strain of *A. versicolor* (L.S.H.T.M. Cat. no. 247) grown on Czapek–Dox medium was exhaustively extracted with light petroleum and then with ether. The solid separating from the ether was purified by crystallization from $CHCl_3$ and *product* B was thus obtained as long orange needles, m.p. 233–234°, unchanged after further recrystallization; $[\alpha]_{5790}^{22}$ −178° in ethanol (*c*, 0·37); light-absorption max. 228, 266, 294, 324 and 453 mμ. in ethanol (log ε, assuming mol.wt. 316 as required by $C_{17}H_{16}O_6$, 4·15, 4·14, 4·37, 3·90 and 3·91 respectively). The yield was only 0·8–1·6 g. from 100 flasks. It contained no nitrogen, halogen, sulphur or methoxyl. (Found: C, 63·9, 64·0; H, 5·7, 5·6. $C_{17}H_{16}O_6$ requires C, 64·1; H, 5·7%.) It is insoluble in water but soluble in cold ethanol, hot $CHCl_3$ and hot benzene. It gives a red colour with ethanolic $FeCl_3$, carmine with cold conc. H_2SO_4 and purple-red with 2N-NaOH. It dissolves in aqueous Na_2CO_3 to give a pink-violet solution and is slightly soluble in aqueous bicarbonate giving a pink solution.

Crystalline compounds obtained in attempts to methylate or acetylate the product were isolated only in minute amounts insufficient for analysis.

Identification of mannitol

By extraction of the mycelium of strains L.S.H.T.M. Cat. no. 247 and A 262 with methanol after exhaustive extraction with (*a*) light petroleum and (*b*) ether, a brown solid was obtained from each strain. This product, on recrystallization from methanol, separated as colourless needles, m.p. 166°. Its m.p. was undepressed on admixture with an authentic sample of D-mannitol. A portion of the product was converted into the hexa-acetate of m.p. 126°. There was no depression in m.p. on admixture with an authentic sample of D-mannitol hexa-acetate, m.p. 126°.

SUMMARY

1. Sterigmatocystin, a crystalline metabolic product obtained from the mycelium of *Aspergillus versicolor* (Vuillemin) Tiraboschi by extraction with light petroleum was isolated as pale-yellow needles of m.p. 247°, $[\alpha]_D^{20}$ −398°. It contains one methoxyl group and possesses the molecular formula $C_{18}H_{12}O_6$.

2. The following derivatives of sterigmatocystin were prepared; diacetate (hydrate), m.p. 228°, monomethyl ether, m.p. 271°, monoethyl ether, m.p. 261°.

3. On fusion with potassium hydroxide sterigmatocystin yields resorcinol and acetic acid, and on zinc-dust distillation small amounts of anthracene or a homologue.

4. When heated with 25% ethanolic potassium hydroxide sterigmatocystin furnishes an optically inactive isomer, *iso*sterigmatocystin, m.p. 234°, yielding a diacetate, m.p. 197°, and a methyl ether, m.p. 162°.

5. A second optically active crystalline product (*B*), m.p. 233°, was obtained in small yield from the mycelium by ether extraction.

6. Mannitol was extracted by methanol from the mycelium as a third metabolic product.

The ultraviolet-absorption spectra were determined with a Uvispek spectrophotometer purchased by means of a grant from the Central Research Fund of London University. We thank Imperial Chemical Industries Ltd., The Frythe, Welwyn, for carrying out the elementary analysis of the bromo compound from sterigmatocystin.

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Studies in the Biochemistry of Micro-organisms

100. METABOLITES OF *PENICILLIUM ATROVENETUM* G. SMITH. PART I. ATROVENETIN, A NEW CRYSTALLINE COLOURING MATTER*

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Penicillium atrovenetum G. Smith is a new species which was described recently by Mr George Smith of this Department (1956). Its specific name was given because colonies of the mould on Czapek agar are at first bright bluish green (*venetus*) in colour but rapidly turn greyer and become almost black (*ater*) in age. The reverse of the colonies is a deep yellowish brown. This species is closely related morphologically to *P. herquei* Bainier & Sartory and should therefore be placed in the *P. herquei* series of the Biverticillata-Symmetrica as described by Raper & Thom (1949). Smith (1956) proposes, however, that a better placement of the *P. herquei* series is in the Asymmetrica-Velutina in close association with the *P. citrinum* and *P. brevi-compactum* series.

During the routine chemical examination of a large number of species of moulds it was found here that the mycelium of the type strain and also of two different strains of *P. atrovenetum*, grown on Czapek–Dox glucose–mineral salts solution, produced some interesting reactions. Immersion of portions of the pressed mycelium in ethanol yielded an orange-brown extract which gave an intensely dark-olive brown, almost black ferric reaction. Other portions immersed in cold concentrated sulphuric acid gave an immediate yellow to orange-brown solution with a strong green fluorescence. We have isolated the fungal colouring matter which is responsible for these colour reactions and, since it has not been described previously, we propose for it the name atrovenetin.

For the bulk preparation of atrovenetin *P. atrovenetum* strain S.M. 683 was cultivated on Czapek–Dox glucose solution for 21 days at 24° in the dark. The mould mycelium was then separated, washed with water, dried *in vacuo* and ground to a fine powder. The powder was extracted with light petroleum to remove 'fat' and then with ether. From the ether extracts, initially very dark green in colour, crude atrovenetin separated as a dark powder from which much of the dark material was separated by extraction with acetone. The residual yellow-brown colouring matter was purified by crystallization from dioxan or acetone. The yield of crystalline atrovenetin obtained averaged about 23 g. from 100 flasks containing 35 l. of Czapek–Dox solution and constituted about 43% of the total ether extract. The combined weights of the light petroleum and ether extracts amounted to 18–20% of the dry weight of the mould mycelium.

Atrovenetin, $C_{19}H_{18}O_6$, forms yellow-brown plates or prisms, which melt with decomposition at 295° and sublime in high vacuum at about 210°. Solutions of atrovenetin in dioxan are strongly dextrorotatory. The molecule of atrovenetin contains a minimum of four hydroxyl groups, no methoxyl group and at least three or probably four methyl groups attached to carbon. It is not readily soluble in the usual organic solvents. It is not soluble in sodium carbonate or sodium bicarbonate solution but dissolves readily in aqueous sodium hydroxide to an orange solution. Its solution in cold concentrated sulphuric acid is bright yellow in colour with a characteristic intense yellow-green fluorescence. It also gives a characteristic intense red-brown ferric reaction in ethanol solution.

The following functional derivatives of atrovenetin have been prepared: atrovenetin hydrochloride, $C_{19}H_{18}O_6.HCl$, yellow needles, m.p. 285–286° (decomp.); atrovenetin perchlorate, $C_{19}H_{18}O_6.HClO_4,2H_2O$,

* Part 99: Birkinshaw & Hammady (1957).

yellow needles, m.p. 227° (decomp.); triacetylatrovenetin, $C_{19}H_{15}O_3.(O.CO.CH_3)_3$, orange-brown cubes, m.p. 187–188°, dextrorotatory and giving a red-brown ferric reaction.

Several methyl ethers of atrovenetin have been prepared, including a number of isomers which are clearly of importance in considering its molecular constitution. They are:

(i) Atrovenetin monomethyl ether A,

$$C_{19}H_{17}O_5(OCH_3),$$

orange-red plates, m.p. 262–263°, which gives a diacetate, $C_{19}H_{15}O_3(OCH_3).(O.CO.CH_3)_2$, golden needles, m.p. 218–219°, and a diethyl ether, $C_{19}H_{15}O_3(OCH_3).(OC_2H_5)_2$, yellow needles, m.p. 129–130°. All three compounds are dextrorotatory and give intense red-brown ferric reactions, but whereas the monomethyl ether A is soluble in aqueous 2N sodium hydroxide, its diacetate and its diethyl ether do not dissolve in this reagent.

(ii) Atrovenetin monomethyl ether B,

$$C_{19}H_{17}O_5(OCH_3),$$

yellow plates, m.p. 226–227°, soluble in aqueous sodium bicarbonate and giving a green ferric reaction.

(iii) Atrovenetin yellow trimethyl ether, $C_{19}H_{15}O_3(OCH_3)_3$, yellow plates, m.p. 168–169°, which gives a ferrichloride, $C_{22}H_{24}O_6.HFeCl_4$, red-orange rhombs, m.p. 148° (decomp.), and a monoacetate perchlorate,

$$C_{19}H_{14}O_2(OCH_3)_3.(O.CO.CH_3).HClO_4,$$

orange-yellow plates, m.p. 185° (decomp.), which does not give a ferric reaction. The parent yellow trimethyl ether is readily soluble in concentrated hydrochloric acid.

(iv) Atrovenetin orange trimethyl ether, $C_{19}H_{15}O_3(OCH_3)_3$, orange plates, m.p. 178–179°, which gives a ferrichloride, $C_{22}H_{24}O_6.HFeCl_4$, red-orange rhombs, m.p. 151–152°, lowered to 125–130° on admixture with the ferrichloride of atrovenetin yellow trimethyl ether, m.p. 148°. Both the trimethyl ethers are dextrorotatory, insoluble in aqueous sodium hydroxide and give an intense red-brown ferric reaction.

(v) Atrovenetin tetramethyl ether A,

$$C_{19}H_{14}O_2(OCH_3)_4,$$

yellow needles, m.p. 141–143°, dextrotatory. It is readily hydrolysed to atrovenetin orange trimethyl ether by boiling with dilute ethanolic hydrochloric acid.

(vi) Atrovenetin tetramethyl ether B,

$$C_{19}H_{14}O_2(OCH_3)_4,$$

yellow prisms, m.p. 186–187°. Both the tetramethyl ethers are insoluble in aqueous sodium hydroxide, but whereas the tetramethyl ether A gives no ferric reaction, the tetramethyl ether B gives a very pale-brown ferric colour.

A number of degradation experiments have been carried out on atrovenetin and on some of its functional derivatives, the results of some of which indicate that atrovenetin is an unexpectedly stable substance. Thus it was recovered substantially unchanged when it was boiled in an atmosphere of nitrogen for a long time either with aqueous 2N sulphuric acid or with aqueous N potassium hydroxide. It also proved stable towards boiling dilute methanolic sulphuric acid, and its yellow and orange trimethyl ethers similarly resisted the action of boiling 10% methanolic hydrochloric acid or 10% methanolic potassium hydroxide. Even when atrovenetin was fused with potassium hydroxide at 300° for 15 min., 60% of it was recovered unchanged. This behaviour is in marked contrast to that of two other fungal colouring matters, herqueinone and norherqueinone, to which, as will be shown later, atrovenetin is related chemically. Norherqueinone, $C_{19}H_{18}O_7$, and its monomethyl ether herqueinone, $C_{20}H_{20}O_7$, have recently been isolated from strains of *Penicillium herquei* Bainier & Sartory (Stodola, Raper & Fennell, 1951; Harman, Cason, Stodola & Adkins, 1955; Galarraga, Neill & Raistrick, 1955). Both these substances are readily decomposed with boiling aqueous 2N sulphuric acid giving one molecule of methyl *iso*propyl ketone, $C_5H_{10}O$, from each substance, together with one molecule of norxanthoherquein, $C_{14}H_{10}O_7$, from norherqueinone and one molecule of xanthoherquein, $C_{15}H_{12}O_7$, from herqueinone. Alkaline hydrolysis of herqueinone leads to deep-seated decomposition, acetaldehyde being the only recognizable decomposition product.

Attempts to dehydrogenate atrovenetin yellow trimethyl ether either with a palladium–charcoal catalyst in boiling Dowtherm in an atmosphere of carbon dioxide or with chloranil in boiling xylene failed, as also did attempts to reduce the same ether either with sodium in boiling ethanol or catalytically with palladium–charcoal–hydrogen. The ether was recovered substantially unchanged in all cases. Reduction experiments with lithium aluminium hydride in ether proved more successful, the two isomeric trimethyl ethers of atrovenetin, $C_{22}H_{24}O_6$, giving different but isomeric reduction products, $C_{22}H_{24}O_5$, i.e. yellow needles, m.p. 145–146°, from the yellow trimethyl ether and scarlet plates, m.p. 203–204°, from the orange trimethyl ether. Neither of the $LiAlH_4$ reduction products gives a positive ferric reaction in ethanol, in contrast to the intense red-brown ferric reactions of the parent methyl ethers.

Treatment of atrovenetin with alkaline hydrogen peroxide at room temperature gave two colourless oxidation products: *A*, $C_{15}H_{16}O_7$, needles, m.p. 188°,

which titrated as a dibasic acid and gave only a faint-brown ferric reaction; *B*, $C_{12}H_8O_7$, needles, m.p. 151–152°, which titrated as a tribasic acid and gave a red-brown ferric reaction. Neither product gave a precipitate with Brady's reagent. Oxidation of atrovenetin with concentrated nitric acid at 100° gave a product, probably $C_{15}H_{14}O_9N_2 + H_2O$, cream rectangular plates, m.p. 234–235°, which, on treatment with diazomethane, gave a monomethyl derivative, probably $C_{16}H_{16}O_9N_2$, cream plates, m.p. 251°. Both these substances are dextrorotatory and both give a negative ferric reaction in ethanol. Only oxalic acid was isolated from the reaction products obtained when atrovenetin was oxidized with alkaline potassium permanganate at room temperature.

Oxidation of atrovenetin yellow trimethyl ether, $C_{22}H_{24}O_6$, with potassium permanganate in boiling acetone gave $C_{22}H_{22}O_8$, pale-yellow needles, m.p. 306–307°, soluble in sodium bicarbonate and giving a red-brown ferric colour. The substance, which still contains three methoxyl groups, appears to be derived from the parent trimethyl ether by the oxidation of a C-CH_3 group to C-CO_2H. Oxidation of the yellow trimethyl ether at 100° with chromic acid in acetic acid gave a substance $C_{19}H_{16}O_6$, yellow-brown prisms, m.p. 277–278°, which contains only one methoxyl group and gives a crystalline oxime, $C_{19}H_{19}O_6N$, in brown-orange needles, m.p. 221°. Attempts to oxidize the trimethyl ether with selenium dioxide by protracted boiling in either acetic anhydride or pentanol led to recovery of the ether in yields of 32 and 86% respectively, but no degradation product was isolated.

Degradation of atrovenetin by heating with electrolytic zinc dust led to the isolation in small yield (3 mg. from 3 g. of atrovenetin) of a colourless crystalline solid which gave an ultraviolet-absorption spectrum that is indistinguishable from that of pure pyrene, $C_{16}H_{10}$, and a crystalline picrate which did not depress the melting point of authentic pyrene picrate on admixture with it.

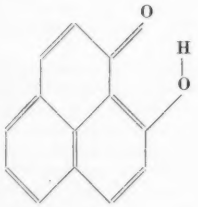
There is no doubt that from the morphological point of view *P. atrovenetum* belongs to the *P. herquei* series of species of *Penicillium* and is closely related to that species (Smith, 1956). This similarity is also evident from the chemical viewpoint. Thus, when the dried mycelium of strains of either *P. atrovenetum* or of *P. herquei* is extracted with ether or chloroform, a very dark-green extract of crude colouring matters is obtained in each case. This is particularly noteworthy since, whereas solvent extracts of a variety of colours may be obtained from different species of *Penicillium*, the production of a green ethereal or chloroform extract is surprisingly rare. Almost equally unusual is the fact that an intensely fluorescent green extract is obtained when the dried mycelium of either *P. herquei* or *P. atrovenetum* is immersed in cold concentrated sulphuric acid.

Hence it is perhaps not surprising that the empirical formulae of the pure colouring matters are so similar, i.e. atrovenetin, $C_{19}H_{18}O_6$, norherqueinone, $C_{19}H_{18}O_7$, and its methyl ether herqueinone, $C_{19}H_{17}O_6(OCH_3)$. Their physical and chemical properties are also very similar in many respects. They are all highly coloured, dextrorotatory, high-melting crystalline solids with definitely similar ultraviolet-absorption spectra. Each of them has a minimum of three and probably four methyl groups attached to carbon. They are all readily soluble in concentrated hydrochloric acid and in dilute aqueous sodium hydroxide to strongly coloured solutions, but none of them dissolves in aqueous sodium carbonate. They all give intense ferric colours in ethanol which, of course, differ in shade. Their solutions in cold concentrated sulphuric acid are yellow to reddish orange in colour with, in each case, an intense yellow-green to green fluorescence. None of them reacts with Brady's reagent. Of special significance appears to be the fact that, on methylation with dimethyl sulphate and anhydrous potassium carbonate in acetone, atrovenetin and herqueinone give, in each case, a mixture of two isomeric trimethyl ethers.

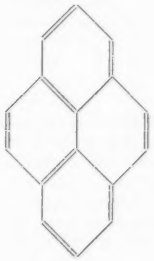
Conclusive evidence has recently been obtained (Neill & Raistrick, 1956; Barton, de Mayo, Morrison, Schaeppi & Raistrick, 1956) showing the close structural relationship of atrovenetin to the *P. herquei* colouring matters. The latter workers reduced norherqueinone, $C_{19}H_{18}O_7$, with zinc dust in acetic acid at room temperature and obtained deoxynorherqueinone, $C_{19}H_{18}O_6$, in the same way as Galarraga *et al.* (1955) obtained deoxyherqueinone, $C_{20}H_{20}O_6$, from herqueinone, $C_{20}H_{20}O_7$, and proved that deoxynorherqueinone and atrovenetin are identical and give identical triacetates.

It has been mentioned previously that, in contrast to the marked stability of atrovenetin to boiling 2N sulphuric acid, both norherqueinone and herqueinone are readily hydrolysed by this reagent, giving respectively norxanthoherquein and its monomethyl ether xanthoherquein, together with a molecule of methyl *iso*propyl ketone in each case. Barton *et al.* (1956) compared the ultraviolet-absorption spectra of xanthoherquein tetraacetate, norxanthoherquein pentaacetate, deoxyherqueinone diacetate and deoxynorherqueinone triacetate (= atrovenetin triacetate), all of which give a positive ferric reaction in ethanol, and expressed the view that all these compounds are derivatives of 9-hydroxyperinaphthen-1-one (I) (Koelsch & Anthes, 1941; Loudon & Razdan, 1954). They further postulated that the structure of atrovenetin may be represented as structure (I) with the addition of three phenolic hydroxyl groups and one CH_3

group attached to carbon, together with one ethereal oxygen probably bound up in the grouping $-CMe_2-CHMe-O-$ and attached, as a ring, to the aromatic system. The formation of pyrene (II) or a close derivative of it on zinc-dust distillation of atrovenetin is then to be explained by scission of the ethereal linkage, followed by recyclization.



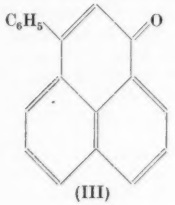
(I)



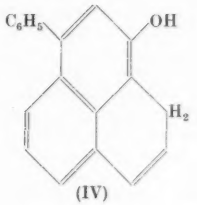
(II)

Until recently very few synthetic, and no naturally occurring, hydroxyperinaphthenones had been described. However, Cooke & Segal (1955*a*) reported a unique red crystalline glycoside, haemocorin, which they obtained from the bulbous roots of the Australian plant, *Haemodorum corymbosum* Vahl. Haemocorin is readily hydrolysed with dilute acid to cellobiose and a purple-red aglycone, $C_{20}H_{14}O_4$, which contains one methoxyl group and gives a diacetate and isomeric mono- and di-methyl ethers. From a study of the functional derivatives, degradation products and absorption spectra of the aglycone, Cooke & Segal (1955*b*) concluded that the aglycone is a dihydroxymethoxyphenylperinaphthenone and suggest that it is probably either 2:6-dihydroxy-5-methoxy-7-phenyl- or 4:9-dihydroxy-3-methoxy-5-phenyl- or 2:4-dihydroxy-5-methoxy-7-phenyl-perinaphthen-1-one. Comparison of the properties of the haemocorin aglycone, atrovenetin, xanthoherquein and norxanthoherquein reveals some noteworthy resemblances. Thus all these compounds, in common with 9-hydroxyperinaphthen-1-one, show a hydrogen-bonded and conjugated carbonyl group in the infrared between 1620^{-1} and 1630^{-1} cm. Similarly, the haemocorin aglycone gives two pairs of isomeric methyl ethers, i.e. monomethyl ethers A and B and dimethyl ethers A and B; atrovenetin gives three pairs, i.e. monomethyl ethers A and B, trimethyl ethers A and B and tetramethyl ethers A and B. Cooke & Segal (1955*b*) synthesized 4-hydroxyperinaphthen-1-one and 6-hydroxy-4-phenylperinaphthen-1-one and showed that each of these compounds gave two isomeric methyl ethers. Finally the dimethyl ether A of haemocorin aglycone and atrovenetin tetramethyl ether A are readily partially demethylated by boiling with very dilute ethanolic mineral acid, i.e. sulphuric or hydrochloric acid, giving in the latter case atrovenetin trimethyl ether.

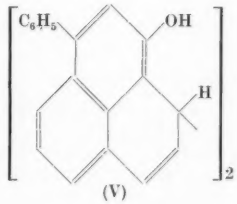
Koelsch & Anthes (1941) showed that the yellow 3-phenylperinaphthen-1-one (structure III) is readily reduced with zinc dust and acetic acid in an atmosphere of hydrogen to give a colourless product of structure (IV) which is readily oxidized in air. A ligroin solution of (III) reacts with an equivalent amount of (IV) in the same solvent to give a quantitative yield of the quinhydrone (V) which is deep red in colour. Since many examples are known among fungal metabolic products of the simultaneous occurrence in fungal cultures of the quinonoid and quinol forms of such products, and even of the quinhydrone in some cases, we believe that the intense green colour of extracts of the mycelium of *P. atrovenetum* and of *P. herquei* may well be due to the quinhydrones of structures, analogous to (V), of atrovenetin on the one hand and of herqueinone or norherqueinone on the other hand.



(III)



(IV)



(V)

EXPERIMENTAL

Cultures

Atrovenetin has been isolated from each of the following strains of *Penicillium atrovenetum* G. Smith:

(*a*) The type strain (L.S.H.T.M. Cat. no. S.M. 683) was isolated here in May 1952 by Mrs S. Marcus from soil from the Sussex Downs.

(*b*) Strain S.M. 715 was isolated here in June 1952 by Mrs S. Marcus from soil from the sand dunes at Perranporth, Cornwall.

(*c*) Strain M. 467 was isolated here in 1943 by Mrs J. M. Webb from the soil of a mangold field in Norfolk.

A detailed description of the species is given by Smith (1956).

Cultural conditions and characteristics

Czapek–Dox solution (glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; water, 1000 ml.) was distributed in 350 ml.

amounts in 1 l. conical flasks. The flasks were plugged with cotton wool and sterilized. The contents of the flasks were then inoculated with a spore suspension in water prepared from Czapek–agar slopes of the strain of *P. atrovenetum* under investigation, three flasks being sown from each slope. The flasks were incubated in the dark at 24°.

During the incubation period the mycelium of strain S.M. 683, which was used for the bulk preparation of atrovenetin, became very folded. The upper surface, which after 7 days incubation was a bright bluish green colour, became much darker after 21 days and showed a few yellowish green pitted areas. The reverse surface, initially brownish orange in colour, gradually darkened to olive brown. The culture fluid was lemon yellow in colour after 7 days, becoming brownish orange after 21 days. Cultures of strains S.M. 715 and M. 467 were very similar in appearance to those of strain S.M. 683.

At intervals during the incubation period portions of the mycelium of strain S.M. 683 were pressed as dry as possible between filter papers. Treatment of some of this material with much cold conc. H_2SO_4 gave an immediate lemon-yellow solution with a strong green fluorescence after 7 days, increasing in intensity after 14 and 21 days incubation. Extraction of other portions of the dried mycelium with ethanol gave highly coloured solutions increasing from orange brown to very intense dark brown with age and giving most striking ferric reactions, increasing from dark brown after 7 days to almost black after 21 days incubation. The mycelium of strains S.M. 715 and M. 467 gave similar but less intense reactions.

A number of cultural details of representative batches of the three strains of *P. atrovenetum*, including yields of atrovenetin, are collected in Table 1. The five batches of strain S.M. 683 are representative of twelve batches harvested over a period of 2½ years and are arranged in chronological order.

Isolation of the mycelial colouring matters

At the end of the incubation period the mycelium from each strain was separated, by straining through muslin, from the culture fluid, which was discarded after measuring its pH and glucose content by polarimeter. The mycelium was washed with water, squeezed as dry as possible in a tincture press and dried in a vacuum oven at 40–45°. The dried mycelium (Table 1, column 6) was finely ground in a coffee mill and extracted for two working days in a large copper Soxhlet with light petroleum (b.p. 40–60°). The extract, on concentration, gave a brown oil (Table 1, column 7). The extracted mycelium was re-dried and was then re-extracted in the Soxhlet with anaesthetic ether for 8 days when the extract, which was initially very dark green in colour, had become almost colourless. The solid crude colouring matter which separated was collected each day and dried. The total combined crude colouring matter (Table 1, column 8) was finely ground and intimately mixed with about an equal volume of clean sand and the mixture was Soxhlet-extracted with acetone.

The yellow-brown colouring matter which separated from the acetone extracts from *P. atrovenetum* strain S.M. 683 consisted of crystalline atrovenetin. It was collected frequently and separated into two main fractions: (*a*) m.p. 288° (decomp.) or above which was usually pure enough for the preparation of derivatives, and (*b*) m.p. usually between 260° and 270° (decomp.). The weights and m.p.'s of these fractions are given in Table 1, columns 9 and 10. Fraction (*b*) was further purified by crystallization from dioxan, from which solvent atrovenetin separated with dioxan of crystallization which was removed by immersing the crystals in a little cold acetone, when they slowly effloresced to yield solvent-free atrovenetin, m.p. 285° and above.

Strain M. 467 gave a good yield of crude atrovenetin, 6·25 g. from thirty-five flasks, which although of low m.p., 263° (decomp.), had almost the same optical rotation as pure atrovenetin from strain S.M. 683, i.e. $[\alpha]^{22}_{5461}+150°$, $[\alpha]^{22}_{6790}+116°$, in dioxan (*c*, 1·000). Its identity was confirmed by conversion into the perchlorate, orange-yellow needles, m.p. 230° (decomp.) (see p. 171); the hydrochloride, yellow needles, m.p. 288° (decomp.) (see p. 171); and the two isomeric methyl ethers by methylation with diazomethane, i.e. the yellow trimethyl ether, m.p. 169° and mixed m.p. 168–169°, with material prepared from strain S.M. 683, and the orange trimethyl ether, m.p. 177–179° and mixed m.p. 178–179° (see p. 172).

The ether extract from strain S.M. 715 contained a much smaller proportion of atrovenetin than strains S.M. 683 or M. 467. The ether extract (10·35 g.) consisted of a very

Table 1. *Cultural details and yields of crude atrovenetin from strains of* Penicillium atrovenetum *G. Smith*

Strain no.	Incubation period (days)	No. of flask cultures	Final pH of culture medium	Residual glucose by polarimeter (%)	Wt. of dried mycelium (g.)	Light petroleum extract 'fat' (g.)	Ether extract (g.)	Yields of crystalline atrovenetin	
								g.	M.p.
S.M. 683	28	32	4·5	0·25	129	3·7	19·02	8·8	280°
S.M. 683	21	100	4·6	0·39	396	22·2	45·7	4·3	292
								5·0	290
								10·2	260
S.M. 683	21	150	4·6	0·68	573	27·1	90·4	25·8	288
								15·8	285
S.M. 683	21	149	4·0	0·61	532	—	76·6	2·95	292
								36·2	263
								5·1	257
S.M. 683	21	148	4·0	0·55	542	16·3	84·0	22·0	290
S.M. 715	21	50	4·9	0·36	190	15·2	10·35	0·70	260
M. 467	28	35	4·0	1·31	91	—	—	6·25	263

dark-green solid from which only 0·7 g. of crude atrovenetin was obtained after trituration with acetone. Portions of this material were converted into the perchlorate, 0·1 g. giving 0·103 g. of perchlorate, m.p. 228° (decomp.) and not depressed on admixture with atrovenetin perchlorate, m.p. 230° (decomp.) from strain S.M. 683. The presence of atrovenetin was confirmed by methylating a second portion with diazomethane, which gave the yellow trimethyl ether of atrovenetin, m.p. 162–163° (see p. 172), not depressed on admixture with a specimen, m.p. 168–169° from strain S.M. 683.

Purification and general properties of atrovenetin

Atrovenetin was purified for analysis either by crystallization from acetone as yellow-brown rectangular plates, or from dioxan as yellow-brown prisms containing solvent of crystallization, or by sublimation in high vacuum at 210° as yellow-orange rectangular plates, the m.p. of all three specimens being 295° (decomp.). (Found, on specimen from strain S.M. 683: C, 66·8; H, 5·3; N, S, Cl, nil; *C*-CH_3, 8·4, 9·5; OCH_3, nil; on specimen from strain M. 467: C, 66·8; H, 5·6. $C_{19}H_{18}O_6$ requires C, 66·7; H, 5·3; 2*C*-CH_3, 8·8%.) $[\alpha]_{5461}^{21}$ +154°, $[\alpha]_{5790}^{21}$ +116° in dioxan (*c*, 0·486). Owing to the relatively slight solubility of atrovenetin and its derivatives in organic solvents and the intense colour of the solutions obtained, the determination of their optical rotations is difficult and is subject to a large error.

Atrovenetin is not readily soluble in the usual organic solvents but dissolves in hot acetone, acetic acid and dioxan. Its golden solution in dioxan has an intense yellow-green fluorescence. It dissolves in cold aqueous 2N-NaOH to give an orange solution which changes reversibly on warming to a deep red colour. It does not dissolve in aqueous Na_2CO_3. It gives a yellow solution in cold conc. H_2SO_4 with an intense yellow-green fluorescence. Its ethanolic solution gives an intense red-brown colour with ethanolic $FeCl_3$. It does not react with Brady's reagent for carbonyl groups (0·32% 2.4-dinitrophenylhydrazine in aqueous 2N-HCl), nor does it give any colour on reduction with magnesium and HCl or with sodium amalgam followed by acidification. It slowly gives a blue colour with 2:6-dibromoquinoneimide (Gibbs, 1927).

Atrovenetin readily gives a water-stable boroacetate as follows: a mixture of atrovenetin (0·2 g.), boroacetic anhydride (0·3 g.) (Dimroth & Faust, 1921; Dimroth, 1926) and acetic anhydride (1·5 ml.) was refluxed for 10 min. On cooling, the bright orange-yellow solid which separated was collected, washed well with ether and dried. It did not melt below 360° and was recovered unchanged after refluxing with water for 1 hr.

Functional derivatives of atrovenetin

Atrovenetin hydrochloride. Atrovenetin (0·2 g.) was suspended in warm acetic acid (20 ml.). Conc. HCl (2 ml.) was added to the mixture, when atrovenetin immediately dissolved to give an orange-yellow solution from which *atrovenetin hydrochloride* crystallized on cooling and was recrystallized from the same solvent mixture as yellow needles (0·23 g.), m.p. 285–286° (decomp.). (Found, on sample dried in high vacuum at room temp.: C, 60·3; H, 5·2; Cl, 9·5. $C_{19}H_{18}O_6$.HCl requires C, 60·2; H, 5·1; Cl, 9·4%.) The hydrochloride decomposes on heating at 220° in high vacuum giving a chlorine-free sublimate of atrovenetin, m.p. 292°.

Atrovenetin perchlorate. Perchloric acid (1 ml. of 60% (w/v)) was added to a suspension of atrovenetin (0·2 g.) in hot acetic acid (5 ml.). The pigment dissolved and, on cooling, yellow needles (0·15 g.), m.p. 225° (decomp.) separated and were collected and crystallized from ethyl acetate giving *atrovenetin perchlorate dihydrate* as yellow needles, m.p. 230° (decomp.). (Found: C, 47·3; H, 4·9; Cl, 7·3. $C_{19}H_{18}O_6.HClO_4.2H_2O$ (=$C_{19}H_{23}O_{12}Cl$) requires C, 47·65; H, 4·8; Cl, 7·4%.)

Triacetylatrovenetin. A mixture of atrovenetin (0·5 g.), acetic anhydride (5 ml.) and anhydrous sodium acetate (0·1 g.) was heated on the boiling-water bath for 2 hr. The orange-brown solid obtained when the acetylation mixture was poured into ice–water (200 ml.) was collected, washed with water and dried. The dried solid was extracted with cold ether. The ether extract was concentrated, giving a crystalline solid on standing, m.p. 183–186°, recrystallization of which from methanol to constant m.p. gave *triacetylatrovenetin* as orange-brown cubes (0·14 g.), m.p. 187–188°. The yellow methanolic solution of the acetate has an intense yellow-green fluorescence and gives a red-brown ferric reaction. (Found: C, 63·8; H, 5·3; $CH_3.CO$, 28·3. $C_{25}H_{24}O_9$ requires C, 64·1; H, 5·2; $3CH_3.CO$, 27·6%.) $[\alpha]_{5461}^{22}$ +104°, $[\alpha]_{5790}^{22}$ +84° in acetic acid (*c*, 1·00). Acetylation of atrovenetin with acetic anhydride and $HClO_4$ as catalyst at room temp., or with sodium acetate or pyridine as catalyst under reflux, led to products which could not be crystallized.

Atrovenetin monomethyl ethers A and B

Atrovenetin monomethyl ether A. A solution of diazomethane in ether prepared from nitrosomethylurea (10 g.) was added to a suspension of atrovenetin (5 g.) in methanol (30 ml.) and ether. The pigment quickly dissolved and when the initially vigorous reaction had moderated (about 3 min.) an orange crystalline solid separated (1·4 g.), m.p. 248–250° and was collected by filtration. (For treatment of the filtrate see p. 172.) The substance was recrystallized from a large volume of acetone giving *atrovenetin monomethyl ether A* (1·27 g.) as orange-red rectangular plates, m.p. 262–263°. (Found: C, 67·4, 67·4; H, 5·65, 5·74; N, nil; OCH_3, 9·0; *C*-CH_3, 9·2. $C_{20}H_{20}O_6$ requires C, 67·4; H, 5·7; 1 OCH_3, 8·7; 2*C*-CH_3, 8·4%.) $[\alpha]_{5461}^{21}$ +224°, $[\alpha]_{5790}^{21}$ +176° in $CHCl_3$ (*c*, 0·504). The ether is soluble in aqueous 2N-NaOH to an orange solution but does not dissolve in aqueous Na_2CO_3. It dissolves in cold conc. H_2SO_4 to a yellow solution with an intense yellow-green fluorescence. Its ethanolic solution gives an intense red-brown ferric reaction.

Diacetylatrovenetin monomethyl ether A. A mixture of atrovenetin monomethyl ether A (0·3 g.), acetic anhydride (5 ml.) and anhydrous sodium acetate (0·2 g.) was heated at 100° for 8 hr. The acetic anhydride was removed under reduced pressure and the residue was crystallized from methanol giving *diacetylatrovenetin monomethyl ether A* as glistening golden needles (0·135 g.), m.p. 218–219°. (Found: C, 65·5; H, 5·6; OCH_3, 6·8; $CH_3.CO$, 20·5. $C_{24}H_{24}O_8$ requires C, 65·45; H, 5·5; 1 OCH_3, 7·0; $2CH_3.CO$, 19·5%.) $[\alpha]_{5461}^{22}$ +105°, $[\alpha]_{5790}^{22}$ +101° in dioxan (*c*, 0·501). The substance is not soluble in aqueous 2N-NaOH. Its methanolic solution has an intense yellow-green fluorescence and gives an intense red-brown ferric reaction.

Diethyl ether of atrovenetin monomethyl ether A. A mixture of atrovenetin monomethyl ether A (0·5 g.) dry acetone (50 ml.), anhydrous K_2CO_3 (2 g.) and diethyl sulphate (0·6 ml.) was refluxed for 3 hr. Additional diethyl sulphate (0·4 ml.) and K_2CO_3 (1 g.) were then added and refluxing was continued for a total of 8 hr. The ethylation product (0·27 g.) was worked up in the usual way and purified by crystallization from methanol, giving the *diethyl ether of atrovenetin monomethyl ether A* as yellow needles, m.p. 129–130°. The substance is not soluble in aqueous 2N-NaOH but its ethanolic solution gives an intense red-brown ferric reaction. [Found: C, 69·9; H, 6·9; alkoxyl as OCH_3, 22·8. $C_{24}H_{28}O_8$ ($=C_{19}H_{18}O_6+CH_2+2C_2H_4$) requires C, 69·9; H, 6·8; three alkoxyl groups calculated as 3 OCH_3, 22·6%.] $[\alpha]^{21}_{5461}+128°$, $[\alpha]^{21}_{5790}+104°$ in ethanol (*c*, 0·497).

Atrovenetin monomethyl ether B. A mixture of atrovenetin (0·3 g.) in aqueous 20% NaOH (20 ml.) and dimethyl sulphate was shaken in the cold, the reaction being maintained alkaline. A pale-yellow crystalline sodium salt, m.p. 340–341° (decomp.) separated and was collected (0·11 g.). It was suspended in dilute HCl, extracted with ether and the extract was evaporated to dryness. The residue was crystallized from aqueous methanol giving *atrovenetin monomethyl ether B* as yellow plates, m.p. 226–227°. (Found: C, 67·1; H, 5·7; OCH_3, 8·3. $C_{20}H_{20}O_6$ requires C, 67·4; H, 5·7; 1 OCH_3, 8·7%.) This ether is soluble in $NaHCO_3$ to a yellow solution, and in cold conc. H_2SO_4 to a yellow solution with an intense yellow-green fluorescence. It gives a green ferric reaction in ethanol and a negative reaction with Brady's reagent.

Atrovenetin trimethyl ethers—yellow and orange

(*a*) *Yellow trimethyl ether.* The ethereal diazomethane filtrate from the preparation of atrovenetin monomethyl ether A was kept overnight at 0° and the excess of diazomethane was destroyed with acetic acid. The ether solution was then washed successively with aqueous N-NaOH, N-HCl and water, dried ($MgSO_4$) and evaporated to dryness. The crystalline residue was washed with a little methanol to remove gum. This residue (2·26 g.) was repeatedly crystallized from methanol giving *yellow atrovenetin trimethyl ether* as bright-yellow rectangular plates, m.p. 168–169°. (Found: C, 68·4; H, 6·4; OCH_3, 24·25; *C*-CH_3, 11·8; mol.wt., cryoscopic in camphor, 361. $C_{22}H_{24}O_6$ requires C, 68·7; H, 6·3; 3 OCH_3, 24·2; 3*C*-CH_3, 11·7%; mol.wt. 384.) $[\alpha]^{21}_{5461}+124°$, $[\alpha]^{21}_{5790}+122°$ in methanol–$CHCl_3$ (*c*, 1·036).

The yellow methyl ether is soluble in ether, $CHCl_3$, acetone, and in hot methanol or ethanol, the ethanolic solution having a yellow-green fluorescence and giving an intense red-brown ferric reaction. The ether is readily soluble in conc. HCl giving a yellow solution, and its solution in cold conc. H_2SO_4 has an intense yellow-green fluorescence. It is not soluble in aqueous 2N-NaOH but when sodium methoxide is added to a methanolic solution of the ether the sparingly soluble sodium salt separates as pale-yellow plates, m.p. 344° (decomp.). (Found: C, 64·5; H, 5·5; Na, 5·7. $C_{22}H_{23}O_6Na$ requires C, 65·0; H, 5·7; Na, 5·7%.) The methyl ether does not react with 2:4-dinitrophenylhydrazine nor does it give any colour on reduction with magnesium and HCl or with sodium amalgam followed by acidification. It may also be prepared, though less readily, by methylating atrovenetin with dimethyl sulphate or methyl iodide and K_2CO_3 in acetone.

Ferrichloride of yellow trimethyl ether. A solution of atrovenetin yellow trimethyl ether (0·1 g.) in conc. HCl (1 ml.) was mixed with a solution of $FeCl_3$ (0·2 g.) in conc. HCl (2 ml.). The resulting orange precipitate was collected, dried and crystallized from acetic acid containing $FeCl_3$ and conc. HCl. The *ferrichloride* (88 mg.) forms deep-red orange rhombs, m.p. 148° (decomp.). (Found: C, 45·6; H, 4·5; Fe, 9·3; OCH_3, 15·85. $C_{22}H_{24}O_6.HFeCl_4$ requires C, 45·3; H, 4·3; Fe, 9·6; 3 OCH_3, 16·0%.)

Acetate perchlorate of yellow trimethyl ether. Perchloric acid (60%; 12 drops) was added to a suspension of the yellow trimethyl ether (0·5 g.) in acetic anhydride (3 ml.). The mixture became warm and the ether dissolved. The solution was heated at 100° for 5 min. On cooling, the orange solid which slowly separated was collected, dried and crystallized from methanol giving the *acetate perchlorate* (0·46 g.) as orange-yellow plates, m.p. 185° (decomp.), the m.p. varying with the rate of heating. (Found: C, 54·3; H, 5·3; Cl, 6·55; OCH_3, 17·7. $C_{24}H_{26}O_7.HClO_4$ requires C, 54·7; H, 5·2; Cl, 6·7; 3 OCH_3, 17·7%.) $[\alpha]^{22}_{5461}+148°$, $[\alpha]^{22}_{5790}+132°$ in acetic acid (*c*, 0·500). An ethanolic solution of this compound has an intense yellow-green fluorescence but gives no ferric reaction. All attempts failed to acetylate the yellow trimethyl ether with acetic anhydride and anhydrous sodium acetate, or pyridine, or conc. H_2SO_4, as catalyst, the starting material being the only crystalline product recovered.

(*b*) *Orange trimethyl ether.* This ether has been prepared in three ways.

(i) The methanol mother liquors and washings from the methylation of atrovenetin with diazomethane were combined and evaporated to dryness. The residue was fractionally crystallized from methanol and was separated by hand sorting into further amounts of the yellow trimethyl ether together with a small amount of an orange isomer. This was recrystallized from methanol giving the *orange trimethyl ether* (0·28 g.) as orange rectangular plates, m.p. 178–179°, mixed m.p. with the yellow trimethyl ether 139–142°. (Found: C, 68·55; H, 6·25; OCH_3, 24·2. $C_{22}H_{24}O_6$ requires C, 68·7; H, 6·3; 3 OCH_3, 24·2%.) $[\alpha]^{22}_{5461}+138°$, $[\alpha]^{22}_{5790}+112°$ in ethanol (*c*, 0·505).

(ii) Atrovenetin monomethyl ether A (0·1 g.), m.p. 262–263°, suspended in methanol, was methylated for 36 hr. at 0° with excess of ethereal diazomethane. The ether solution was then washed successively with 2N-NaOH, N-HCl and water, dried ($MgSO_4$) and evaporated to dryness. The residue was crystallized from methanol giving orange rectangular plates (0·04 g.), m.p. and mixed m.p. with the orange trimethyl ether prepared as in (i) above, 178–179°.

(iii) A mixture of atrovenetin monomethyl ether A (2 g.), dry acetone (600 ml.), dimethyl sulphate (1·17 ml., 2·2 moles) and anhydrous K_2CO_3 (2 g.) was refluxed for 3 hr. Additional K_2CO_3 (2 g.) and dimethyl sulphate (0·9 ml.) were added and heating was continued for a further 3 hr. The methylation product was worked up in the usual way and crystallized from methanol giving the orange trimethyl ether (1·73 g.), m.p. and mixed m.p. 178–179°.

The orange trimethyl ether is soluble in ether, $CHCl_3$, acetone and in hot methanol and ethanol. It does not dissolve in aqueous 2N-NaOH but gives an intense red-brown ferric reaction in ethanol. It could not be acetylated with sodium acetate and acetic anhydride.

Ferrichloride of orange trimethyl ether. This derivative was prepared and crystallized in the same way as the ferri-

chloride of the yellow trimethyl ether; yield 0·14 g. from 0·1 g. of ether. It crystallizes in deep-red orange rhombs, m.p. 151–152°; mixed m.p. with the ferrichloride of the yellow trimethyl ether, 125–130°. (Found: C, 45·3; H, 4·4; Fe, 9·25; OCH_3, 15·8. $C_{22}H_{24}O_6.HFeCl_4$ requires C, 45·3; H, 4·3; Fe, 9·6; 3 OCH_3, 16·0%.)

Atrovenetin tetramethyl ethers A and B

A (i). From atrovenetin yellow trimethyl ether. A mixture of the yellow trimethyl ether (1 g.), sodium-dried benzene (120 ml.), methyl iodide (20 ml.) and freshly precipitated and dried Ag_2O (2 g.) was heated under reflux. Further Ag_2O was added at 2-hourly intervals, i.e. 4 × 1 g., followed by 4 × 0·5 g., and refluxing was continued for a total of 24 hr. Solvents were then removed by evaporation and the residue was extracted with ether. The ether extract, evaporated to 20 ml., was chromatographed on Al_2O_3 (2·5 cm. × 14 cm.) which was then eluted with ether in 100 ml. fractions. The first and second fractions (200 ml.) of eluate were combined, the solvent was removed and the residue was washed with light petroleum (b.p. 40–60°). The residual yellow crystalline solid (0·29 g.), m.p. 131–134°, was crystallized from aqueous methanol giving *atrovenetin tetramethyl ether A* (0·21 g.) as yellow needles, m.p. 141–143°. (Found: C, 69·2; H, 6·8; OCH_3, 30·0. $C_{23}H_{26}O_6$ requires C, 69·3; H, 6·6; 4 OCH_3, 31·15%.) $[\alpha]^{21}_{5461}$ +156°, $[\alpha]^{21}_{5790}$ +126° in ethanol (*c*, 1·000).

A (ii). From atrovenetin orange trimethyl ether. The orange trimethyl ether was methylated and the product was purified in the same way as is described in section (i) immediately above; yield 0·14 g. from 0·5 g. of trimethyl ether. Yellow needles, m.p. 139–140°; mixed m.p. 140–141° with the tetramethyl ether A (m.p. 141–143°) described above. (Found: C, 69·4; H, 6·7; OCH_3, 30·2. $C_{23}H_{26}O_6$ requires C, 69·3; H, 6·6; 4 OCH_3, 31·15%.) $[\alpha]^{21}_{5461}$ +146°, $[\alpha]^{21}_{5790}$ +122° in ethanol (*c*, 0·994).

Identical ultraviolet- and infrared-absorption spectra were given by the two specimens of atrovenetin tetramethyl ether A described in sections (i) and (ii) above.

Atrovenetin tetramethyl ether A does not dissolve in aqueous 2N-NaOH and gives no ferric reaction in ethanolic solution. Its yellow solutions in cold conc. H_2SO_4 and in ethanol have an intense yellow-green fluorescence. It is readily soluble in most organic solvents except light petroleum.

Atrovenetin tetramethyl ether A was readily partially demethylated to atrovenetin orange trimethyl ether as follows. A solution of the ether (60 mg.) in ethanol (10 ml.) containing conc. HCl (0·3 ml.) was refluxed for 4 hr. Ethanol was then removed by evaporation and the residue was neutralized with aqueous $NaHCO_3$ and extracted with ether. The dried (Na_2SO_4) extract was evaporated and the crystalline residue (43 mg.), m.p. 173–175°, was crystallized from methanol giving atrovenetin orange trimethyl ether as orange rectangular plates (25 mg.), m.p. 174–175°, mixed m.p. with an authentic specimen 176–178°. The product also gave an intense red-brown ferric reaction in ethanol.

B. From atrovenetin yellow trimethyl ether. The third and fourth fractions (200 ml.) of ether eluate from section (A i) above were combined and evaporated, and the residue was crystallized from light petroleum (b.p. 40–60°) as yellow cubes (41 mg.), m.p. 181–182°. Recrystallization from aqueous methanol gave *atrovenetin tetramethyl ether B* as yellow prisms, m.p. 186–187°. (Found: C, 69·3; H, 6·6; OCH_3, 30·7. $C_{23}H_{26}O_6$ requires C, 69·3; H, 6·6; 4 OCH_3, 31·15%.) This ether is insoluble in aqueous 2N-NaOH and gives a definite brown or red-brown ferric reaction in ethanol which is discharged on the addition of water. Its yellow solution in cold conc. H_2SO_4 has a yellow fluorescence.

Degradation experiments with atrovenetin and its derivatives

A. Acid hydrolysis experiments. A mixture of atrovenetin [1 g., m.p. 295° (decomp.)] in aqueous 2N-H_2SO_4 (150 ml.) was refluxed for 11 hr. in a slow stream of O_2- and CO_2-free Na_2. The issuing N_2 was passed first through bubblers containing Brady's reagent and then through standard $Ba(OH)_2$ solution. No precipitate was formed in the Brady solution and only traces of $BaCO_3$ were precipitated. The mixture was then distilled to about 30 ml., water (100 ml.) was added and the distillation was repeated. The combined distillates, neutral in reaction, gave no precipitate with Brady's reagent. The solid in the H_2SO_4 suspension was collected, washed with water and dried, wt. 0·976 g., m.p. 285° (decomp.). It was shown to be substantially unchanged atrovenetin by methylation in ether–methanol with ethereal diazomethane (see p. 171), when atrovenetin monomethyl ether A (36 mg.), m.p. and mixed m.p. 256°, atrovenetin yellow trimethyl ether (0·32 g.), m.p. and mixed m.p. 164–165°, and atrovenetin orange trimethyl ether (54 mg.), m.p. and mixed m.p. 179–180°, were obtained.

Atrovenetin was also recovered unchanged (0·42 g.), m.p. and mixed m.p. 289° (decomp.), after a solution of it (0·5 g.) in methanol (50 ml.) containing conc. H_2SO_4 (0·75 g.) was boiled for 6 hr.

B. Alkaline hydrolysis experiments. A solution of atrovenetin (1 g.) in aqueous N-KOH (100 ml.) was refluxed for 48 hr. in a slow stream of purified N_2. The effluent N_2 was passed through bubblers containing standard $Ba(OH)_2$ solution. The KOH solution was cooled and acidified through a tap funnel with 4N-H_2SO_4, the stream of N_2 being continued. Titration of the $Ba(OH)_2$ solution showed that only traces of CO_2 had been formed. The orange solid precipitated in the acidified KOH was collected (0·952 g.) and a portion of it was methylated with diazomethane giving atrovenetin yellow trimethyl ether as yellow rectangular plates, m.p. and mixed m.p. 168–169°.

The yellow and orange trimethyl ethers of atrovenetin were recovered unchanged in 68–92% yield after refluxing for 3–8 hr. either in 10% methanolic KOH or in 10% methanolic HCl.

C. Potash-fusion experiments. Atrovenetin (1 g.) was added in portions to a stirred melt of KOH (10 g.) and water (1 ml.) at 210°. The temp. was then slowly raised to 300° and held there for 15 min. The cooled melt was dissolved in water (100 ml.) and acidified with H_2SO_4. The resulting orange precipitate was collected and extracted with ether. Evaporation of the ether extract gave an orange-brown crystalline solid (0·6 g.), m.p. 292° (decomp.), which gave the usual colour reactions of atrovenetin, sublimed in high vacuum at 210° in yellow-brown rectangular plates, m.p. 293° (decomp.) and gave atrovenetin yellow trimethyl ether, m.p. and mixed m.p. 168–169°, on methylation with diazomethane.

Variations in the temp. of the melt, held for 2 hr. in each case, led to the following results: 325–335°, 0·563 g. of recovered pigment, m.p. 292–294°; 330–340°, 0·16 g. of

pigment, m.p. 284–286° (decomp.); 340–350°, complete decomposition. The acid filtrates from all these experiments were combined and continuously extracted with ether. The only recognizable breakdown product isolated from the ether extract was oxalic acid (82 mg.).

Fusion of atrovenetin yellow trimethyl ether (0·5 g.) with KOH (10 g.) and water (1 ml.) for 15 min. at 280–300° yielded only resinous products.

D. Reduction experiments. (i) $LiAlH_4$ *reduction of the atrovenetin trimethyl ethers.* (*a*) *Atrovenetin yellow trimethyl ether.* A percolator containing this trimethyl ether (3 g.) was attached to a reflux condenser and the whole was connected with a reaction flask containing sodium-dried ether (approx. 450 ml.) and a slurry of $LiAlH_4$ (1·5 g.). The mixture was refluxed for 0·5 hr. after all the trimethyl ether had been extracted. Excess hydride was decomposed with ethyl acetate, and $Al(OH)_3$ was dissolved by the addition of aqueous NaOH. The ether layer was separated, washed with water, dried and evaporated to dryness. The residue was dissolved in benzene and chromatographed on alumina.

The main *reduction product YA*, which was obtained from the later eluates, was purified by crystallization from methanol giving yellow needles (1·34 g.), m.p. 145–146°. (Found: C, 71·7, 71·6; H, 6·7, 6·5; OCH_3, 24·7. $C_{22}H_{24}O_5$ requires C, 71·7; H, 6·6; 3 OCH_3, 25·3%.) $[\alpha]_{5461}^{26}+276°$, $[\alpha]_{5790}^{26}+206°$ in ethanol (*c*, 1·000). This reduction product resists acetylation at 100° with acetic anhydride and sodium acetate.

(*b*) *Atrovenetin orange trimethyl ether.* A solution of this ether (1 g.) in dry ether (250 ml.) was added to a slurry of $LiAlH_4$ (about 0·5 g.) in dry ether, and the mixture was refluxed for 0·5 hr. The reduction product was worked up and chromatographed in benzene on Al_2O_3 as described in section (*a*) above. Eluates 3, 4 and 5 gave a product which, on crystallization from methanol, yielded *reduction product OA* as scarlet prisms (0·1 g.), m.p. 203–204°. (Found: C, 71·8, 72·0; H, 6·9, 7·1; OCH_3, 24·6. $C_{22}H_{24}O_5$ requires C, 71·7; H, 6·6; 3 OCH_3, 25·3%.) $[\alpha]_{5461}^{20}+200°$, $[\alpha]_{5790}^{20}+156°$ in dioxan (*c*, 1·000).

Neither of the reduction products gives a ferric reaction. Neither of them is soluble in aqueous 2N-NaOH, nor extractable from $CHCl_3$ solution by this reagent.

(ii) *Catalytic reduction.* A solution of atrovenetin yellow trimethyl ether (0·202 g.) in acetic acid (5 ml.) was shaken for 5 hr. with an active palladium–charcoal catalyst in an atmosphere of H_2. The total absorption of H_2 was 4·4 ml. at 18° and 760 mm. The catalyst was then separated by filtration, the filtrate evaporated to dryness *in vacuo* and the residue crystallized from methanol, giving unchanged yellow trimethyl ether (0·175 g.), m.p. and mixed m.p. 167–169°.

(iii) *With sodium in ethanol.* In parallel experiments solutions of the yellow and orange trimethyl ethers of atrovenetin (0·5 g. in each case) in ethanol (50 ml.) were refluxed, and metallic sodium (0·5 g.) was added in portions. From the reaction mixtures 0·48 g. of the yellow ether and 0·287 g. of the orange ether, recrystallized in each case from methanol, were recovered.

E. Dehydrogenation experiments. (i) A mixture of atrovenetin yellow trimethyl ether (0·5 g.), 5% palladium–charcoal catalyst (0·05 g.) and Dowtherm (10 ml.) was refluxed for 16 hr. in a slow stream of CO_2. The reaction mixture was then diluted with light petroleum (b.p. 40–60°) and filtered. The filtrate was extracted with conc. HCl and the HCl extract was diluted with much water. The resultant yellow precipitate was collected, dried and crystallized from methanol, giving unchanged atrovenetin yellow trimethyl ether (0·335 g.), m.p. and mixed m.p. 164–165°.

(ii) A mixture of atrovenetin yellow trimethyl ether (0·3 g.; 1 mole), chloranil (0·4 g.; 2 moles) and xylene (10 ml.) was refluxed for 16 hr. The cooled reaction mixture was diluted with ether, washed with aqueous 2N-NaOH, dried (Na_2SO_4) and evaporated. The crystalline residue was washed with a little cold methanol and collected; wt. 0·19 g., m.p. 163–165° undepressed on admixture with atrovenetin yellow trimethyl ether.

F. Oxidation experiments on atrovenetin. (i) *With alkaline* H_2O_2. Hydrogen peroxide (30%; 20 ml.) was added slowly to a solution of atrovenetin (1 g.) in aqueous 2N-NaOH (80 ml.). The mixture became green, and after 2 hr. further H_2O_2 (20 ml.) and 10N-NaOH (10 ml.) were added. The mixture was kept overnight at room temp., filtered and the pale straw-coloured filtrate was acidified. The resulting colourless crystalline precipitate (64 mg.), m.p. 176°, was collected, dried and crystallized from benzene–light petroleum (b.p. 60–80°), giving *oxidation product A* as colourless needles, m.p. 188°. The substance gave a faint-brown ferric reaction and did not give a precipitate with Brady's reagent. (Found, on material dried *in vacuo* at 100°: C, 58·85; H, 5·23; equivalent by titration, 156. $C_{15}H_{16}O_7$ requires C, 58·43; H, 5·23%; equiv. (dibasic), 154·1.)

The acid filtrate from oxidation product A was extracted with ether, and the ether solution was extracted with $NaHCO_3$. Acidification and ether extraction of the $NaHCO_3$ solution gave a colourless, partly crystalline gum (0·46 g.) which gave a wine-red ferric colour but no reaction with Brady's reagent. The gum was sublimed in high vacuum at 130–150° and the sublimate of colourless rosettes was purified by crystallization from ether–light petroleum (b.p. 60–80°) and resublimation giving *oxidation product B* (51 mg.) as colourless rosettes of needles, m.p. 151–152°. The product gave a red-brown ferric reaction. (Found: C, 54·4; H, 2·86; equiv. by titration, 90·4. $C_{12}H_8O_7$ requires C, 54·6; H, 3·05%; equiv. (tribasic), 88·1.)

(ii) *With conc.* HNO_3. A mixture of atrovenetin (1 g.) and conc. HNO_3 (5 ml.) was heated at 100° until the evolution of nitrous fumes ceased. It was then cooled, diluted with water and extracted with ether. The ether solution was extracted with aqueous $NaHCO_3$, which was acidified, and the precipitated solid was collected and crystallized from ethyl acetate–light petroleum (b.p. 60–80°); wt. 163 mg., m.p. 234–235°. The *oxidation product* was further crystallized thrice from water containing a few drops of HCl, giving cream rectangular plates, m.p. 239–240°; $[\alpha]_{5461}^{22}+376°$, $[\alpha]_{5790}^{22}+332°$ in acetic acid (*c*, 1·00). (Found, on different samples: C, 46·9, 46·9; H, 4·23, 4·20; N, 6·3; *C*-CH_3, 9·9. $C_{15}H_{16}O_{16}N_2$, i.e. $C_{15}H_{14}O_9N_2+H_2O$, requires C, 46·9; H, 4·20; N, 7·3; 3*C*-CH_3, 11·7%.) The substance does not give a positive fluorescein test. It gives no colour with $FeCl_3$ in ethanol.

The oxidation product (180 mg.) was dissolved in a methanol–ether mixture, and an ether solution of diazomethane was added to it. A colourless solid separated immediately, and after 5 min. the excess of diazomethane was destroyed and the solid was collected, wt. 123 mg., m.p. 250–252°. Recrystallization from ethyl acetate gave the *methyl derivative of the oxidation product* as cream rectangular plates, m.p. 251°. It is only slowly soluble in

dilute aqueous NaOH and gives no colour with $FeCl_3$; $[\alpha]_{5461}^{21} + 226°$, $[\alpha]_{5790}^{21} + 204°$ in dioxan (*c*, 1·00). (Found: C, 50·6; H, 4·38; N, 6·6; OCH_3, 7·95. $C_{16}H_{16}O_9N_2$, i.e. $C_{15}H_{14}O_9N_2 + CH_2$, requires C, 50·5; H, 4·24; N, 7·4; 1 OCH_3, 8·2%.)

G. Oxidation experiments on atrovenetin yellow trimethyl ether. (i) *With* $KMnO_4$. A boiling solution of atrovenetin yellow trimethyl ether (1 g.) in acetone (30 ml.) was oxidized with $KMnO_4$ in acetone until the oxidation proceeded slowly. The mixture was then evaporated to dryness and the residue was suspended in water and saturated with SO_2, giving a yellow-orange precipitate which was collected by filtration. The filtrate was extracted with ether from which only oxalic acid was recovered. The yellow-orange precipitate was dissolved in ether and partitioned between $NaHCO_3$, Na_2CO_3 and NaOH. The extracted ether solution was evaporated to dryness and the residue on crystallization from methanol gave unchanged atrovenetin yellow trimethyl ether (80 mg.).

The $NaHCO_3$ extract was acidified and the yellow precipitate was collected and washed with ether, giving a yellow crystalline solid (45 mg.), m.p. 290°. Recrystallization from methanol gave the *oxidation product* as pale-yellow needles (36 mg.), m.p. 306–307°. (Found: C, 63·8; H, 5·21; OCH_3, 24·1; *C*-CH_3, 7·7. $C_{22}H_{22}O_8$ requires C, 63·75; H, 5·35; 3 OCH_3, 22·5; 2*C*-CH_3, 7·3%.) The substance gives a red-brown ferric colour in ethanol It dissolves only slowly in $NaHCO_3$ but is readily soluble in Na_2CO_3.

The Na_2CO_3 and NaOH extracts gave only negligible amounts of material.

(ii) *With* CrO_3. A solution of CrO_3 (0·5 g.) in water (3 ml.) and acetic acid (20 ml.) was added during 1 hr. to a solution, heated at 100°, of atrovenetin yellow trimethyl ether (0·5 g.) in acetic acid (10 ml.). The solution was held at 100° for a further hour. The resulting green solution was evaporated *in vacuo* giving a partially crystalline residue which was diluted with methanol, and the solid (112 mg.), m.p. 263–264°, was collected. Crystallization from acetic acid–water or $CHCl_3$–light petroleum (b.p. 60–80°) gave the CrO_3 *oxidation product* as yellow-brown prisms, m.p. 277–278°. (Found: C, 66·5, 66·6; H, 5·16, 5·26; OCH_3, 9·2. $C_{19}H_{18}O_6$ requires C, 66·7; H, 5·30; 1 OCH_3, 9·1%.) The compound was recovered unchanged after heating for 2 hr. at 100° with acetic anhydride and anhydrous sodium acetate. It gives a precipitate with Brady's reagent.

A solution of the oxidation product (50 mg.) and hydroxylamine hydrochloride (50 mg.) in pyridine (about 1 ml.) was heated at 100° for 2 hr. and was then poured into water. The product was crystallized from ethyl acetate giving the *oxime of the oxidation product* (11 mg.) as brown-orange rosettes of needles, m.p. 221°. (Found: C, 63·5; H, 5·45. $C_{19}H_{19}O_6N$ requires C, 63·8; H, 5·36%.)

(iii) *With selenium dioxide.* Mixtures of the trimethyl ether (0·5 g. in each case) with SeO_2 (0·2 g. freshly sublimed) were boiled in (*a*) acetic anhydride (5 ml.) for 6 hr. and (*b*) pentanol (10 ml.) for 24 hr. Unchanged trimethyl ether was recovered in 32·6% yield in case (*a*) and in 86·6% in case (*b*). The mother liquors gave positive reactions with Brady's reagent in each case, but no crystalline carbonyl compound could be isolated.

H. Zn dust-distillation of atrovenetin. An intimate mixture of finely ground atrovenetin (3 g.) and electrolytic zinc dust (150 g.) was distributed among 150 special glass tubes similar in construction to those described by Kögl & Postowsky (1924). The tubes were heated to a dull-red heat and the crystalline distillates which collected in the capillary end of the tubes were extracted with ether. After removal of the solvent, the residue was dissolved in light petroleum (b.p. 40–60°) and chromatographed on Al_2O_3, the separation being observed in u.v. light. The more rapidly moving and blue-fluorescing fraction I was collected, rechromatographed and sublimed twice *in vacuo* giving a colourless crystalline solid (3 mg.), m.p. 125–128°. It gave a crystalline picrate with ethanolic picric acid which, on recrystallization from ethanol, formed long glistening red needles, m.p. 218–220° and mixed m.p. 218–220° with authentic pyrene picrate, m.p. 222°. Its u.v.-absorption spectrum is indistinguishable from that of pyrene.

The more slowly eluted yellow band was rechromatographed and sublimed twice *in vacuo* giving fraction II as a pale-yellow solid (0·64 mg.), the ethanolic solution of which fluoresced blue.

Ultraviolet-absorption spectra

The u.v.-absorption spectra of atrovenetin, herqueinone and norherqueinone and a number of their derivatives were determined by means of a Hilger and Watts 'Uvispek' spectrophotometer with the collaboration of Dr J. H. Birkinshaw. The wavelengths (mμ.) and molar extinction coefficients (log ε, in parentheses) of the maxima ($\lambda_{max.}$) and also of the principal minimum ($\lambda_{min.}$) of the curves obtained are assembled below. All absorption spectra were measured in ethanolic solution.

(1) Atrovenetin. λ 222 mμ. (log ε, 4·52); inflexion at 250–260 mμ. (4·26); 385 mμ. (4·21); inflexion at 410–420 mμ. (4·10); $\lambda_{min.}$ 310 mμ. (2·74).

(2) Herqueinone. 220 mμ. (4·29); 250 mμ. (4·09); 314 mμ. (4·47); inflexion at 365–380 mμ. (3·50); 416 mμ. (3·66); $\lambda_{min.}$ 270 mμ. (3·89).

(3) Norherqueinone. 223 mμ. (4·41); inflexion at 251–254 mμ. (4·13); 315 mμ. (4·59); plateau at 360–390 mμ. (3·66); plateau at 410–430 mμ. (3·61); $\lambda_{min.}$ 275 mμ. (3·98).

(4) Xanthoherquein. 222 mμ. (4·47); 387 mμ. (4·23); $\lambda_{min.}$ 310 mμ. (3·31).

(5) Atrovenetin yellow trimethyl ether. 228 mμ. (4·47); inflexion at 270–275 mμ. (4·18); inflexion at 380–400 mμ. (4·31); $\lambda_{min.}$ 297 mμ. (2·51).

(6) Atrovenetin tetramethyl ether B, m.p. 186–187°. 220 mμ. (3·91); 281 mμ. (3·89); 364 mμ. (3·48); 426 mμ. (3·58); $\lambda_{min.}$ 302 mμ. (2·08).

(7) $LiAlH_4$ reduction product YA, m.p. 145–146°. 220 mμ. (4·31); 239 mμ. (4·36); 272 mμ. (4·40); 340 mμ. (3·96); 422 mμ. (4·14); $\lambda_{min.}$ 300 mμ. (2·90).

(8) $LiAlH_4$ reduction product OA, m.p. 203–204°. 220 mμ. (4·48); 275 mμ. (4·56); 342 mμ. (3·97); 438 mμ. (4·27); 462 mμ. (4·25); $\lambda_{min.}$ 295 mμ. (3·00).

(9) Colourless Zn dust distillate of atrovenetin, fraction I, ? pyrene. Inflexion at 230 mμ. (4·55); 240 mμ. (4·70); 262 mμ. (4·35); 272 mμ. (4·55); 287 mμ. (3·98); 306 mμ. (4·00); 318 mμ. (4·31); 334 mμ. (4·51).

(10) Authentic pyrene (Friedel & Orchin, 1951). 231 mμ. (4·65); 241 mμ. (4·9); 262 mμ. (4·4); 273 mμ. (4·7); inflexion at 293 mμ. (3·65); 306 mμ. (4·05); 318 mμ. (4·5); 334 mμ. (4·7).

(11) Yellow Zn dust distillate of atrovenetin, fraction II, not identified. 223 mμ.; 237 mμ.; 257 mμ.; 266 mμ.; 278 mμ.; 289 mμ.; 316 mμ.; 331 mμ.; 365 mμ.; 384 mμ.

Derivatives of xanthoherquein and norxanthoherquein

Xanthoherquein tetraacetate. Xanthoherquein (0·5 g.) was acetylated with acetic anhydride (5 ml.) and 60% $HClO_4$ (5 drops) at 100° for 1 hr. The crude brown acetate obtained was collected, dried (0·78 g.) and extracted with benzene (40 ml.), leaving a bright-yellow solid which was crystallized from ethyl acetate and gave *xanthoherquein tetraacetate* (0·26 g.) as yellow rods, m.p. 217°. (Found: C, 58·1; H, 4·48; OCH_3, 6·6; $CH_3.CO$, 37·6. $C_{23}H_{20}O_{11}$ requires C, 58·5; H, 4·27; 1 OCH_3, 6·6; 4$CH_3.CO$, 36·4%.) The product gives a positive ferric colour in ethanol and dissolves very slowly in aqueous 2N-NaOH to an orange solution, probably owing to hydrolysis. Its yellow solution in cold conc. H_2SO_4 has a yellow-green fluorescence.

Norxanthoherquein pentaacetate. A mixture of norxanthoherquein (0·2 g.), acetic anhydride (2 ml.) and 60% $HClO_4$ (5 drops) was heated at 100° until all of the solid dissolved. The crystalline solid which separated on cooling was collected, washed and crystallized from much ethyl acetate giving *norxanthoherquein pentaacetate* as yellow needles (153 mg.), m.p. 235–236°. (Found: C, 57·5; H, 4·12; $CH_3.CO$, 47·9. $C_{24}H_{20}O_{12}$ requires C, 57·6; H, 4·03; 5$CH_3.CO$, 43·0%.) An ethanolic solution of the product, which gives a positive ferric colour, is yellow with a green fluorescence. The substance is sparingly soluble in the usual organic solvents.

Norxanthoherquein hexamethyl ether. A mixture of norxanthoherquein pentamethyl ether (0·3 g., m.p. 141°), dry benzene (30 ml.), methyl iodide (2 ml.) and freshly prepared Ag_2O (0·2 g.) was refluxed. After 8 hr. further Ag_2O (0·2 g.) and methyl iodide (2 ml.) were added and refluxing was continued for a total of 24 hr. The mixture was then evaporated to dryness and the residue was extracted with ether. The ether extract was concentrated and chromatographed on Al_2O_3. The column was eluted with ether giving a small initial green fraction which was discarded. The solvent was removed from the main eluate and the residue was extracted with light petroleum (b.p. 40–60°) from which, on evaporation, *norxanthoherquein hexamethyl ether* separated as large orange needles (71 mg.), m.p. 109–110°. (Found: C, 64·1; H, 5·87; OCH_3, 49·2. $C_{20}H_{22}O_7$ requires C, 64·15; H, 5·92; 6 OCH_3, 49·7%.) The product gives no ferric colour in ethanolic solution.

SUMMARY

1. The dried mycelium of laboratory cultures of the type strain of *Penicillium atrovenetum* G. Smith has yielded, by solvent extraction, about 15% of its weight of a complex mixture of crude colouring matters from which about half this weight of a hitherto undescribed pigment, now named atrovenetin, has been isolated in a state of purity.

2. Two other strains of *P. atrovenetum* also yield atrovenetin.

3. Atrovenetin, $C_{19}H_{18}O_6$, yellow-orange plates, m.p. 295° (decomp.), is a dextrorotatory substance having characteristic colour reactions.

4. A number of functional derivatives and degradation products of atrovenetin are described.

5. Atrovenetin is closely related structurally to the colouring matters, herqueinone and norherqueinone, of *Penicillium herquei* Bainier & Sartory, and is identical with deoxynorherqueinone.

6. Atrovenetin, herqueinone and norherqueinone are believed to be derivatives of 9-hydroxyperinaphthen-1-one.

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Cacao Polyphenolic Substances

4. THE ANTHOCYANIN PIGMENTS*

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The purple colouring matter of cacao cotyledons has been shown to consist of two cyanidin glycosides (Forsyth, 1952). In that study the pigments were obtained only as amorphous powders, now known to have been grossly contaminated with colourless glucosides. The anthocyanin which moves the faster on chromatography was incorrectly considered to contain both glucose and arabinose, and the slower anthocyanin to contain glucose alone.

Better methods of extraction and purification have now yielded crystalline pigments shown by comparison with synthetic specimens to be 3-α-L-arabinosidyl cyanidin chloride and 3-β-D-galactosidyl cyanidin chloride.

EXPERIMENTAL

Synthetic cyanidin-3-monoglycosides. 3-β-D-Glucosidyl cyanidin chloride (chrysanthemin), 3-β-D-galactosidyl cyanidin chloride (idaein), and 3-β-D-xylosidyl cyanidin chloride were synthesized by the classical route (MacDowell, Robinson & Todd, 1934).

In contrast to the findings of MacDowell *et al.* (1934), no difficulty was experienced in crystallizing the xyloside as chloride nor was it found to be exceptionally unstable to acid. It crystallized as long needles from 5% ethanolic HCl [all alcoholic HCl percentages are for dry HCl gas in the alcohol (w/w)]. (Found on anhydrous pigment: xylose, 33·0. Calc. for monopentoside, 33·0%.)

3-α-L-*Arabinosidyl cyanidin chloride*, which had not hitherto been prepared, is readily synthesized by the usual route, with methods identical with those successful for the preparation of the xyloside. The picrate crystallizes as small dark needles from saturated aqueous picric acid solution at 0°. The chloride is formed by dissolving in 5% methanolic HCl and precipitating with ether. On dissolving this precipitate in 0·05N aqueous HCl and adding an equal volume of 5% ethanolic HCl the arabinosidyl cyanidin chloride crystallizes almost immediately. It is readily recrystallized from the same solvents as almost black prisms, with a green glance when dry. (After drying for 3 hr. at 100° *in vacuo*. Found: C, 53·6; H, 4·1. Calc. for $C_{20}H_{19}O_{10}Cl$: C, 52·8; H, 4·2%.)

Cacao pigments. The beans used were from the clone Imperial College Selection no. 1. Freshly dried cacao cotyledons (1 kg.) are extracted with 0·1N-HCl (8 l.), the extract is saturated with NaCl, and the ethyl acetate-soluble and saline-insoluble phenols are removed, as previously described (Forsyth, 1955). The saline extract remaining is then shaken with *n*-butanol (¼ vol.) and the butanol concentrated under reduced pressure to about 200 ml. The NaCl is removed by filtration and the pigments are precipitated by dilution with 5 vol. of light petroleum (80–100°). The deep-red precipitate is filtered, washed with light petroleum and dried *in vacuo*.

A cellulose-pulp column (Forsyth, 1952) of 40 cm. × 3·7 cm. is of sufficient size to give two well-separated pigment bands when 2 g. of the crude pigments is chromatographed with the top layer of a pentanol–acetic acid–water (4:1:5, by vol.) mixture.

The column is extruded, the individual bands are excised and the pigments separately eluted with methanol. The methanol extracts are concentrated to small volume under reduced pressure and precipitated by dilution with light petroleum (80–100°). The crude pigments are dissolved in a minimal volume of saturated aqueous picric acid and crystallized overnight at 0°. The picrates are centrifuged off and washed with saturated aqueous picric acid, then with ether. They are converted into the chlorides on dissolving in 5% methanolic HCl and precipitating with ether. The chlorides are crystallized by dissolving in 0·05N aqueous HCl, adding an equal volume of 5% ethanolic HCl and allowing to stand at 0°. They can be purified by repeated recrystallization in this manner.

Sugar estimation. The glycosides are hydrolysed with 2N-H_2SO_4 for 30 min. at 100°. The glycosidic sugars present in the cacao bean, i.e. glucose, galactose, arabinose and xylose, are best separated on paper with the top layer of an ethyl acetate–pyridine–water (3:1:3, by vol.) mixture as solvent. They are quantitatively estimated on the paper by a modification of the method of Shu (1950), in which the sugars are not eluted from the paper but paper blanks are used. An internal standard (Flood, Hirst & Jones, 1947) is used.

RESULTS AND DISCUSSION

The faster-moving anthocyanin (3-α-L-*arabinosidyl cyanidin chloride*). When first crystallized this pigment gave on hydrolysis glucose and xylose as well as the main sugar. Recrystallization, however, gave a product yielding only a single sugar. Xylose was the more persistent contaminant, but after four crystallizations it was not generally detectable in the hydrolysate. The single sugar could not be separated from added arabinose in several solvents. The anthocyanidin from the hydrolysis of the pure pigment has the crystalline form, R_F values (Bate-Smith, 1949, 1954), colour reactions (Robinson & Robinson, 1931; Robertson & Robinson, 1929),

* Part 3: Forsyth (1955).

visible- (Scott-Moncrieff, 1930) and ultraviolet-absorption spectra of cyanidin obtained by the hydrolysis of the synthetic 3-α-L-arabinosidyl cyanidin chloride.

As isolated the anthocyanin contained 1 mol. prop. of water of crystallization. (After drying for 3 hr. at 100° *in vacuo*. Found: C, 52·6; H, 3·9; Cl, 7·9. Calc. for $C_{20}H_{19}O_{10}Cl$: C, 52·8; H, 4·2; Cl, 7·8%.)

The arabinose was estimated with rhamnose as an internal standard. [Found (calc. on anhydrous pigments): fast anthocyanin, 33·9, 34·1, 35·0, 35·5; synthetic 3-α-L-arabinosidyl cyanidin chloride, 34·0. Calc. for monopentoside, 33·0%.]

The slower-moving anthocyanidin (*3-β-D-galactosidyl cyanidin chloride*). When first crystallized this pigment gave on hydrolysis glucose and arabinose together with galactose. Several crystallizations were required before the product gave only a single sugar on hydrolysis. The single sugar could not be separated from added galactose in several solvents. The anthocyanidin was cyanidin, as before, but on first crystallizing from the hydrolysed anthocyanin solution its crystalline form was different from that of the cyanidin from the synthetic arabinoside, but identical with that from the synthetic galactoside. As isolated the anthocyanin contained 2·5 mol.prop. of water of crystallization. (After drying for 3 hr. at 100° *in vacuo*. Found: C, 51·6; H, 4·3; Cl, 7·7. Calc. for $C_{21}H_{21}O_{11}Cl$: C, 52·0; H, 4·3; Cl, 7·3%.)

The galactose was estimated with arabinose as internal standard. [Found (calc. on anhydrous pigment): slow anthocyanin, 37·4, 38·1; synthetic 3-β-D-galactosidyl cyanidin chloride, 37·6. Calc. for monohexoside, 37·2%.]

Comparison with synthetic 3-glycosidyl cyanidin chlorides. The two isolated cacao monoglycosides have the sugar in the 3-position since they give identical colour changes as acid-base indicators (Robertson & Robinson, 1929) and have ultraviolet and visible spectra very similar to those of the four synthetic 3-glycosides, which themselves give identical colour reactions and have spectra closely similar to one another. The pentosides are readily distinguished from the hexosides by their R_F values in *n*-butanol–acetic acid (Bate-Smith & Westall, 1950): galactoside, 0·33; slower cacao anthocyanin, 0·33; glucoside, 0·34; arabinoside, 0·41; faster cacao anthocyanin, 0·41; xyloside, 0·43.

On paper chromatography mixtures of the arabinoside and xyloside or galactoside and glucoside (Robinson & Smith, 1955) separate sufficiently (although not into two discrete spots) to show that in such cases two compounds are present. If the glucosidyl and xylosidyl cyanidins are present they can be detected in the presence of the galactosidyl and arabinosidyl compounds. They are not detected in extracts of the cacao bean. The colour intensity of equal concentrations of the aglycone, pentoside and hexoside are in the ratio 1:0·62:0·52, the isolated pigments having the same intensity as the corresponding synthetic compounds. The slower-moving anthocyanin has the crystalline form, both as picrate and chloride, of the corresponding 3-β-D-galactosidyl cyanidin salts, the faster-moving anthocyanin that of the 3-α-L-arabinosidyl cyanidin salts. A few specks of 3-β-D-galactosidyl cyanidin chloride dusted on a saturated ethanolic HCl solution of the slower compound causes immediate crystallization; the same occurs when the faster compound is dusted with 3-α-L-arabinosidyl cyanidin chloride.

Confirmation by enzymic hydrolysis. During the curing of the cacao bean to produce the basic raw material of the chocolate industry the two anthocyanin pigments are destroyed. The first stage is enzymic hydrolysis of the glycosides (Forsyth & Quesnel, unpublished work). A glycosidase preparation of considerable activity and specificity can be prepared from the fresh bean.

Freshly dried cacao cotyledons are fractionated to separate the pigmented cells by sedimentation in light petroleum as described by Brown (1954). The

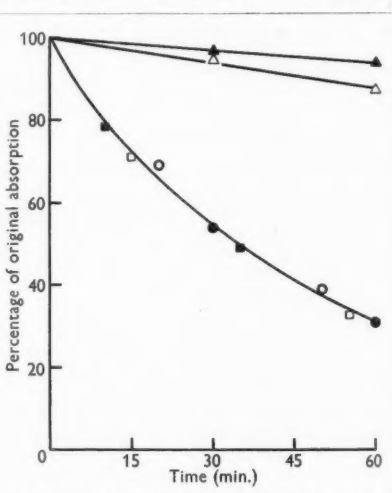

Fig. 1. Enzymic hydrolysis of cyanidin-3-glycosides with cacao-bean preparation (see text) at 44° and pH 4·0 in the absence of air. Anthocyanin [1 mg. in 50% (v/v) ethanol (0·2 ml.)] was present in the top of the Thunberg tube; 2 ml. of enzyme preparation (pH 4·0) was in the bottom. The tubes were evacuated, allowed to equilibrate at 44°, and the contents were mixed. After reaction, the volume was made up to 50 ml. with 0·2 N ethanolic HCl and the absorption read with yellow-green filter, max. transmission 540 mμ. ▲, Xyloside; △, glucoside; ■, galactoside; □, slow-moving cacao anthocyanin; ●, arabinoside; ○, fast-moving cacao anthocyanin.

lighter fraction, mainly free from pigmented cells, is washed with 50% (v/v) aqueous acetone at 0° until free from phenols, and then with 80%, and finally anhydrous, acetone to dry it. The acetone-dried white powder (25 g.) is extracted with 0·2 M-Na barbital buffer (500 ml.), pH 8·0, in a blender, after first passing it as a paste through a triple-roll mill. After centrifuging, the extract is adjusted to pH 4·0 with 0·2 N-HCl, filtered, and 2 vol. of ethanol added to the acid filtrate at 0°. The resultant precipitate is centrifuged off and dissolved in 50 ml. of McIlvaine buffer, pH 4. It is stable at 0° for at least 4 weeks. The activity of such a preparation towards the anthocyanins is shown in Fig. 1. It is necessary to use anaerobic conditions to preclude polyphenol oxidase activity. The hydrolysis is most readily followed by colorimetry. A Spekker photoelectric absorptiometer was used with a 0·5 cm. cell and a yellow-green filter (no. 7 of Kodak set H 769; peak transmission 540 mμ.). Under the conditions used, the resultant cyanidin is mainly, but not entirely, in the colourless pseudo-base form. This accounts for the residual adsorption (Fig. 1), although the sugars are liberated stoicheiometrically as confirmed by direct quantitative chromatography.

It would appear that β-D-galactopyranosyl activity is the major glycosidase function present and that the isolated pigments are indeed 3-β-D-galactosidyl cyanidin chloride and the corresponding 3-α-L-arabinosidyl cyanidin chloride of closely similar structure.

Huang (1956) has used a similar technique with a fungal β-glucosidase to confirm that the anthocyanin pigment of the blackberry is a β-glucoside.

Confirmation by infrared spectra. The infrared spectra of all the 3-monoglycosides were determined with the KBr pellet technique (Li & Wagenknecht, 1956). The results of this analysis confirmed that the slower-moving cacao anthocyanin was indeed the 3-β-D-galactosidyl cyanidin chloride and the faster one the 3-α-L-arabinosidyl cyanidin chloride.

SUMMARY

The anthocyanin pigments of the cacao bean are 3-β-D-galactosidyl and 3-α-L-arabinosidyl cyanidin salts.

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Manganese Oxidation in the Pea Plant (*Pisum sativum* L.) Grown Under Conditions of Manganese Toxicity

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Manganese is known to be an essential micronutrient of plants. It has frequently been suggested that its physiological effects may, at least in part, be due to its capacity for valency change. Kenten & Mann (1949, 1950) showed that Mn^{2+} ions at physiological concentrations are oxidized by peroxidase systems and suggested that *in vivo* manganese is involved in a cycle of oxidation and reduction which may be partly responsible for the stimulating effects of manganese on plant respiration shown by Lundegårdh (1939). But there is as yet no direct evidence that manganese is oxidized in plants grown under normal conditions of manganese supply. If the oxidation of manganese does in fact occur *in vivo* under such conditions, then, as suggested by Kenten & Mann (1952*a*), it might be expected that the oxidation product would be reduced by plant metabolites as rapidly as it was

formed. Under conditions of manganese toxicity, however, the rate of oxidation of Mn^{2+} ions might be more rapid than the rate of reduction of the oxidation product, thus leading to accumulation of the latter. Kelley (1914) obtained evidence of the presence of 'manganese dioxide' in the tissues of plants showing manganese toxicity. The present work was undertaken to investigate whether it was possible to demonstrate the presence of compounds containing manganese of valency greater than two ('higher-valency forms of manganese') in plants grown in water culture at high levels of Mn^{2+} ions.

MATERIALS AND METHODS

Plant material. The seeds were germinated in acid-washed sand and the seedlings obtained were then transferred to the culture medium in the greenhouse. The medium was contained in 300 ml. bottles and four seedlings were grown in each bottle. The medium was changed weekly.

Composition of culture medium. The nutrient solution, in glass-distilled water, contained as macronutrients mM-KH_2PO_4, 4 mM-$Ca(NO_3)_2,4H_2O$, 2 mM-$MgSO_4,7H_2O$ and 6 mM-KNO_3. In addition, H_3BO_3, $ZnSO_4,7H_2O$, $CuSO_4,5H_2O$ and ammonium molybdate were added to give these concentrations: boron, 1 p.p.m.; zinc, 0·1 p.p.m.; copper, 0·04 p.p.m.; molybdenum, 0·02 p.p.m. Iron was added as a solution of 0·5% (w/v) $FeSO_4,7H_2O$ in 0·4% (w/v) tartaric acid. A volume (0·6 ml.) of this solution was added per litre of culture solution once weekly. Sufficient Mn^{2+} ions as $MnSO_4,4H_2O$ were added to give concentrations from 1 to 220 p.p.m.

Estimation of manganipyrophosphate. The method used was based on that of Kenten & Mann (1955) which depends on the fact that solutions of manganipyrophosphate have an absorption maximum at 258 mμ.

Estimation of total manganese. After wet digestion of the plant material the manganese was oxidized with KIO_4 to permanganate, which was estimated colorimetrically.

Spectrophotometry. A Unicam SP. 500 quartz spectrophotometer was used with 1 cm. quartz cells.

Buffers. Phosphate buffers (0·1 M) were prepared from solutions of KH_2PO_4 and KOH. Pyrophosphate buffers (0·1 M) were prepared from solutions of $Na_2H_2P_2O_7$ and $K_4P_2O_7$.

Hydrated manganese dioxide. This was prepared by the method of Heintze & Mann (1949).

EXPERIMENTAL AND RESULTS

Colorimetric evidence of manganese oxidation

With pea plants at high levels of Mn^{2+} ions in the culture medium (220 p.p.m.) a brown colour developed in the stems, more particularly at the nodes, after 1–2 weeks. This generally increased in intensity with time. Similar though less-distinct symptoms were observed at levels of 110, 55 and 22 p.p.m., but at the latter level the symptom was one of brown speckles on the stem. Sections cut from the brown parts of the stem and immersed in benzidine solution (Kenten & Mann, 1952*b*) generally turned blue, which suggested that 'higher-valency forms of manganese' might be present. When the brown tissue was extracted by grinding in a mortar with 0·1 M pyrophosphate, part of the brown material usually went into solution, and on centrifuging the supernatant solution gave a blue colour with benzidine. Manganic manganese dissolves in pyrophosphate at pH 7 and forms a stable manganipyrophosphate. Although MnO_2 is insoluble in neutral pyrophosphate it dissolves when Mn^{2+} ions are also present, to give manganipyrophosphate.

$$Mn^{2+} + MnO_2 + 4H^+ \rightleftharpoons 2Mn^{3+} + 2H_2O. \quad (1)$$

After extraction the tissue generally still remained brown, but when tested with benzidine directly it gave negative or only faintly positive tests. The residual pigment was not dissolved either by pyrophosphate alone or by pyrophosphate containing $MnSO_4$.

Spectrophotometric evidence of manganese oxidation

Kenten & Mann (1955) have shown that solutions of manganipyrophosphate have an absorption at 258 mμ. and have based a method of estimation of manganipyrophosphate on the intensity of this absorption. It was thought therefore that by extracting the plant material with 0·1 M pyrophosphate, pH 7, centrifuging, and measuring the absorption at 258 mμ. the difference between the absorption of extracts of the experimental and control plants (Mn^{2+} ions, 1 p.p.m.) might give a measure of manganese oxidized. Fig. 1 shows the absorption curves of pyrophosphate extracts from plants grown for 4 weeks in media containing Mn^{2+} ions in the concentrations 1, 22, 55 and 220 p.p.m. The curves obtained with the extracts of the experimental plants resembled the absorption curve of manganipyrophosphate (Fig. 2). However, on treating the extracts with hydrazine sulphate (0·1 ml. of 0·1 M/3·5 ml. of extract) only a comparatively small decrease in the absorption at 258 mμ. was observed, although Kenten & Mann (1955) have shown that such treatment with hydrazine completely destroys the absorption at 258 mμ. of manganipyrophosphate solutions. Much of the increased absorption observed with the experimental plants was therefore due to the presence of compounds other than manganipyrophosphate. Nucleoproteins and nucleic acids and their degradation products give similar absorption curves. Tests showed that the absorption curves of yeast nucleic acid, prepared by the method of Holden & Pirie (1955*a*), before and after partial digestion with leaf ribonuclease (a preparation corresponding to fraction *J* of Holden & Pirie, 1955*b*), were unaffected by hydrazine.

Further experiments showed that it was possible to remove much of the absorbing material not affected by the addition of hydrazine by preliminary extraction of the plant material with 0·1 M phosphate buffer, pH 7. The absorption at 258 mμ. of the subsequent pyrophosphate extracts from the plants grown at high levels of Mn^{2+} ions was then often considerably diminished by hydrazine. The leaves and side shoots were removed from the stems and the latter were then weighed and sliced into segments 1–2 mm. long. Samples of the slices (e.g. 0·25 g.) were ground in a mortar with 5 ml. of 0·1 M phosphate, pH 7. The mixture was centrifuged and the residue was again washed on the centrifuge with a further 5 ml. of phosphate. The residue was suspended in 5 ml. of 0·1 M pyrophosphate, pH 7, and extracted at 0° in the dark for about 30 min. It was then centrifuged, and the absorption spectrum of the extracts, after suitable dilution with phosphate, was measured. An extract obtained by the same procedure from plants grown with normal manganese supply (1 p.p.m.) was used in the blank cell. Hydrazine sulphate (0·1 ml. of 0·1 M soln./3·5 ml.) was added to both cells and the absorption at 258 mμ. again measured. Fig. 2 shows that the phosphate extracts had an absorption spectrum similar to that of manganipyrophosphate but with an absorption maximum at about 265 mμ. The absorption of these extracts was not altered by hydrazine. The subsequent pyrophosphate extracts had absorption spectra almost identical with that of manganipyrophosphate with an absorption maximum at 258 mμ. The absorption of the pyrophosphate extracts was considerably diminished by adding hydrazine. The decrease in absorption when hydrazine was added to the pyrophosphate extract was taken as a measure of the manganipyrophosphate present. In the experiment of Fig. 2 this gave a value of 225 μg. of Mn^{3+} ions/g. of tissue. The amount of Mn^{3+} ions found in this and other similar experiments was not increased by adding Mn^{2+} ions to the pyrophosphate used for extraction. This suggests that if the 'higher-valency form of manganese' present in the plant is MnO_2, sufficient Mn^{2+} ions are also present to satisfy the requirements of equation (1).

Attempts were made to eliminate the possibility that the oxidation of manganese by enzyme systems took place during the extraction procedure. Heat treatment of the tissue (10 min. at 100°) before extraction was tried with the object of inactivating

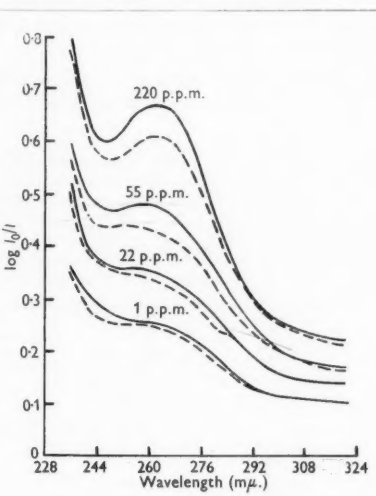

Fig. 1. Absorption curves of the pyrophosphate extracts, before and after hydrazine treatment, of the stems of pea plants grown at varying concentrations of manganese sulphate. Stems were extracted with 10 times their weight of 0·1 M pyrophosphate, pH 7, centrifuged, and the supernatant diluted with 10 times the volume of 0·1 M pyrophosphate, pH 7. Hydrazine sulphate, where added, was 0·1 ml. of 0·1 M soln./3·5 ml. of diluted extract. 0·1 M Pyrophosphate (±hydrazine) was present in the blank cell. —, Without hydrazine; – – –, with hydrazine.

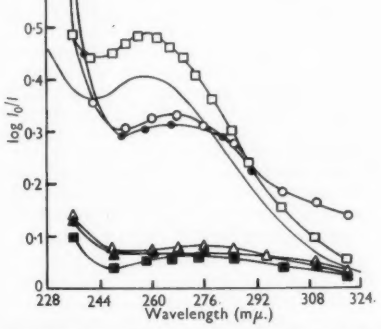

Fig. 2. Absorption curves of consecutive phosphate and pyrophosphate extracts, before and after hydrazine treatment, of the stems of pea plants grown at a concentration of Mn^{2+} ions of 110 p.p.m. for 4 weeks. Similar extracts of control plants (Mn^{2+} ions 1 p.p.m.) with and without hydrazine were used in the blank cell. First phosphate extract diluted with 2 vol. of phosphate: ○, without hydrazine; ●, with hydrazine. Second phosphate extract, undiluted: △, without hydrazine; ▲, with hydrazine. Pyrophosphate extract diluted with 2 vol. of pyrophosphate: □, without hydrazine; ■, with hydrazine. —, Manganipyrophosphate (3·5 μg. of Mn^{3+} ions/ml.) in 0·1 M pyrophosphate, pH 7. Only a few of the experimental points are plotted, for the sake of clarity.

the oxidizing system, and also the effect of adding Mn^{2+} ions to control tissue during the extraction procedure was tried. The results (Table 1) show that when the stems are immersed in boiling water for 10 min. before extraction much less Mn^{3+} ions were found in the pyrophosphate extract. This was probably due to reduction during the heat treatment. When the stems from control plants were ground in a mortar with MnO_2 and phosphate the amount of Mn^{3+} ions obtained by extracting the residue after centrifuging was little less than that obtained when the MnO_2 was similarly treated in absence of tissue, suggesting that only a small fraction of the MnO_2 was reduced to manganous manganese under these conditions. If, however, the macerate of stem, MnO_2 and phosphate was heat-treated before extraction with pyrophosphate the amount of Mn^{3+} ions obtained was considerably less. This suggests that the comparatively low values obtained after heat treatment with plants grown at high levels of Mn^{2+} ions were due to reduction of the 'higher-valency forms of manganese' present. The results (Table 1) provide evidence that the higher oxides found were not formed by oxidation of Mn^{2+} ions during the extraction procedure. Thus when Mn^{2+} ions were added to the stem tissue of control plants, either immediately before the phosphate extraction or to the pyrophosphate used for the final extraction, little or no Mn^{3+} ions were found.

Manometric evidence of manganese oxidation

In plants grown at high levels of Mn^{2+} ions (220 p.p.m.) it was possible to confirm the fact that 'higher-valency forms of manganese' accumulated in the plants by the manometric method of estimation with hydrazine described by Kenten & Mann (1949). In such experiments several stems were taken from the plants grown with toxic concentrations of Mn^{2+} ions and from those grown in the normal media. The two sets of stems were sliced and samples were taken for the manometric and spectrophotometric estimations. In the manometric experiments about 0·5 g. of the slices, suspended in $6{\cdot}7 \times 10^{-2}$ M pyrophosphate, pH 7, were present in each vessel. The vessels were filled with nitrogen. After equilibration 0·2 ml. of a saturated solution of hydrazine sulphate was added from the side arms. In the vessels containing the experimental plant tissue (Mn^{2+} ions, 220 p.p.m.) a gas output was observed. Little or no gas output was observed with the control tissue (Mn^{2+} ions, 1 p.p.m.), either alone or when $MnSO_4$ was added to the vessel contents to give concentrations of Mn^{2+}

Table 1. *Effect of heating the stem tissue in the presence and the absence of added* MnO_2 *on the amount of* Mn^{3+} *ions found in the pyrophosphate extract. Effect of* Mn^{2+} *ions added to the stem tissue immediately before or during the extraction procedure on the amount of* Mn^{3+} *ions found*

Experimental plants were grown at concentrations of Mn^{2+} ions of 1, 55 or 110 p.p.m. In the experiments with added Mn^{2+} ions stem sections of plants grown in culture solution containing 1 p.p.m. were extracted in the usual way, with phosphate followed by pyrophosphate, except that Mn^{2+} ions were added either before the phosphate extraction or during the pyrophosphate extraction. In the heat treatment the stems were cut into sections 2 cm. long and samples (0·3 g.) were immersed in boiling water for 10 min. The usual extraction procedure was then used. In experiments with added MnO_2 (equivalent to 420 μg. of Mn^{3+} ions) the stem samples (0·3 g.) from plants grown at a Mn^{2+} ion concentration of 1 p.p.m. were ground in a mortar with hydrated MnO_2 and 20 times the weight of phosphate, pH 7. The macerates were heated for 10 min. in a boiling-water bath, centrifuged, and the residues washed with phosphate and extracted with pyrophosphate containing 200 μg. of Mn^{2+} ions/ml.

Concentration of Mn^{2+} ions in culture solution (p.p.m.)	Treatment of tissue	Mn^{3+} ions found (μg./g. of tissue)
1	No Mn^{2+} ions added	0
1	140 μg. of Mn^{2+} ions added with phosphate	0
1	550 μg. of Mn^{2+} ions added with phosphate	10
1	140 μg. of Mn^{2+} ions added with pyrophosphate	10
1	550 μg. of Mn^{2+} ions added with pyrophosphate	15
55	Boiled	20
55	Not boiled	85
110	Boiled	20
110	Not boiled	105

Control experiments	Mn^{3+} ions found (μg.)
MnO_2 with tissue (heated)	145
MnO_2 with tissue (not heated)	315
MnO_2 in the absence of tissue (heated)	365
MnO_2 in the absence of tissue (not heated)	370

ions of up to 370 μg./ml. The values for Mn^{3+} ions were always higher by the manometric method than by the spectrophotometric method (Table 2). This was probably due to reduction of the 'higher-valency forms of manganese' during the extraction procedure in the spectrophotometric method of estimation. The small amounts of Mn^{3+} ions found by the manometric method for the plants in Expt. 2 (Mn^{2+} ions, 1 p.p.m.) were almost certainly due to experimental error. No evidence was found either by the spectrophotometric method or by the benzidine test of the presence of 'higher-valency forms of manganese' in such plants.

Table 2. *Spectrophotometric and manometric estimation of 'higher-valency forms of manganese' in the stems of pea plants grown in culture solutions containing* Mn^{2+} *ions* (220 *p.p.m.*)

Expt. no.	Mn^{2+} ions in culture solution (p.p.m.)	Total Mn^{3+} ions found (μg./g. of tissue): Spectrophotometric method	Total Mn^{3+} ions found (μg./g. of tissue): Manometric method
1	220	190	325
	1	0	0
	220	125	225
	1	0	0
2	220	310	315
	1	0	15
	220	315	385
	1	0	25
3	220	250	280
	1	—	0
	220	260	375
	1	—	0

Table 3. *Effect of time of growth and concentration of* Mn^{2+} *ions in the culture solution on the accumulation of 'higher-valency forms of manganese' in the stems of pea plants*

Higher-valency forms of manganese present were estimated by the spectrophotometric method.

Concn. of Mn^{2+} ions in the culture solution (p.p.m.)	Time of growth (weeks)	Mn^{3+} ions found (μg./g. of tissue): Expt. 1	Expt. 2	Expt. 3
11	6	0	—	—
	7	0	—	—
22	2	—	0	—
	3	0	0	—
	4	0	20	—
	5	—	—	—
	6	25	—	—
	7	25	—	—
55	2	0	0	—
	3	0	40	—
	4	0	55	55
	5	30	—	45
	6	20	—	75
	7	55	—	—
110	2	0	10	—
	3	15	55	—
	4	45	250	45
	5	25	—	80
	6	125	—	95
220	2	—	—	—
	3	—	—	—
	4	—	—	275
	5	—	—	160
	6	—	—	—

Effect of time of growth and concentration of Mn^{2+} *ions*

A number of experiments were done in which plants grown at various concentrations of Mn^{2+} ions were tested at weekly intervals for the presence of 'higher-valency forms of manganese'. Some of the results obtained are set out in Table 3. There was a large variation in the content of 'higher-valency forms of manganese' of plants grown under apparently identical conditions. Despite these variations the following conclusions can be drawn. No accumulation of 'higher-valency forms of manganese' was found when the concentration of Mn^{2+} ions in the culture solution was less than 22 p.p.m. At concentrations of 22 p.p.m. upwards the time of appearance of the higher-valency forms and the final concentration reached was, in general, directly related to the concentration of Mn^{2+} ions in the culture solution. Concentrations of Mn^{2+} ions of 55 p.p.m. upwards caused a rapid appearance of chlorosis and stunting of growth. At a concentration of Mn^{2+} ions of 220 p.p.m. growth practically ceased after 2–3 weeks.

Relation between the amounts of total manganese and 'higher-valency forms of manganese' accumulating in the stems

In two experiments both the total manganese and its 'higher-valency forms' present in the stems were estimated. The results are shown in Table 4. In these experiments no 'higher-valency forms of manganese' were found in the stems of the plants grown at a concentration of Mn^{2+} ions of 22 p.p.m., though the total manganese was high in comparison with that of the control stems (Mn^{2+} ions, 1 p.p.m.). With higher levels of Mn^{2+} ions in the culture solution the Mn^{3+} ions found formed a considerable fraction of the total manganese in the stem and in one case more than one-half of the total manganese was accounted for as Mn^{3+} ions.

Injection experiments

Since it is known that certain micro-organisms can bring about the oxidation of Mn^{2+} ions the results so far obtained did not exclude the possibility that the 'manganese higher-valency forms' found were formed by such oxidation in the culture

solutions and then taken up in the oxidized form by the plant. The roots of the plants grown in culture solutions containing higher concentrations of Mn^{2+} ions were dark brown, and sometimes gave a strongly positive benzidine reaction due presumably to 'higher valency-forms of manganese'. As it was not easy to decide how far these were present as a deposit on the root surface rather than inside the roots, the stem tissue was used in the present work. Even when high concentrations of 'higher-valency forms of manganese' were present in the stems the leaves gave negative or only weakly positive benzidine reactions. To exclude the possibility that the 'higher-valency forms of manganese' found in the plants were not derived from the culture solution the oxidation of Mn^{2+} ions was also demonstrated, in experiments in which the Mn^{2+} ions were injected into the plants via the leaf petioles according to the technique of Roach (1945). Although it was not possible with this technique to demonstrate manganese oxidation in the plants at low concentrations of Mn^{2+} ions, 'higher-valency forms of manganese' accumulated in the stems within a few hours when the strength of the Mn^{2+} ions solution injected was 0·05–0·1 M (Table 5). Accumulations of the same order were also found when Mn^{2+} ions were injected into plants kept in the dark.

Table 4. *Relation between the total manganese contents of the stems of pea plants grown for four weeks in water culture at various concentrations of* Mn^{2+} *ions and the amount of 'higher-valency forms of manganese' that accumulated*

Expt. no.	Mn^{2+} ions in culture solution (p.p.m.)	Total Mn (μg./g. of tissue)	Mn^{2+} ions found (μg./g. of tissue)
1	1	5	0
	22	55	0
	55	155	45
	110	540	325
2	1	5	0
	22	40	0
	55	205	50
	110	550	155

Table 5. *Effect of varying the concentration of* Mn^{2+} *ions and the time of injection on the accumulation of 'higher-valency forms of manganese' in the stems of pea plants*

Pea seedlings were grown in soil until they reached a height of about 15 cm., and $MnSO_4$ solutions were then injected through the petioles.

Time of injection (hr.)	Concn. of $MnSO_4$ soln. (M)		
	0·02	0·05	0·1
	Mn^{3+} found (μg./g. of tissue)		
6	—	20	20
24	—	120	135
48	145	410	630
72	180	300	860
96	210	—	—

Results with other plants

A number of other plants [tomatoes (*Lycopersicum esculentum* Mill.), sprouts (*Brassica oleracea* var. *gemmifera* Zenk.) and barley (*Hordeum vulgare* L.)] were grown in water culture with added Mn^{2+} ions under conditions similar to those used with pea plants. The growth of tomatoes and sprouts was much more strongly inhibited by high concentrations of Mn^{2+} ions than that of pea plants, whereas barley survived at high concentrations of Mn^{2+} ions, as is already known from the work of Brenchley (1927). With none of these plants was definite evidence obtained of accumulation of 'higher-valency forms of manganese' in the tissues, although in some cases under toxic concentrations of Mn^{2+} ions browning of the tissues was observed.

DISCUSSION

The present work shows that 'higher-valency forms of manganese' accumulate in pea plants grown under conditions of manganese toxicity. The valency form of the manganese and the precise nature of these accumulations have not yet been established. The accumulations appear to be associated with a browning of the tissues, which may represent a deposition of a higher oxide of manganese with necrosis of the neighbouring tissues. But occasionally with the pea plant and invariably with the other plants, where the browning of the tissues occurred, it was not accompanied by accumulation of demonstrable amounts of 'higher-valency forms of manganese'. These brown areas may be sites where the rate of oxidation of manganese is high enough to bring about a failure of the normal metabolism and to cause necrosis but not sufficiently high to lead to the accumulation of the oxidation product of manganese. The evidence at present available suggests that such accumulation is due to the oxidation of Mn^{2+} ions by peroxidase systems (Kenten & Mann, 1950, 1952*a*), which at high concentrations of Mn^{2+} ions becomes more rapid than the reduction of the oxidation product by plant metabolites. The possibility that accumulation follows a photo-induced oxidation either by the chloroplasts, as suggested by the results of Kenten & Mann (1955), or by a system of the type described by Andreae (1955), would appear to be excluded by the results of the injection experiments in light and darkness. Negative results obtained with other plants investigated may be due to the rate of reduction of the higher-valency forms being as great as the rate of oxidation of Mn^{2+} ions. But these

negative results do suggest that manganese toxicity is not due in all cases to accumulation of 'higher-valency forms of manganese' in the plant tissues. It is of interest that accumulation of ferric iron in the vascular-plate tissues of the nodes of *Zea mays* L., particularly under conditions of potassium deficiency, was observed by Hoffer & Trost (1923) and Hoffer (1930).

The present results show that a valency change of manganese does occur in plants *in vivo* and support the hypothesis that the function of manganese as an essential element in plant nutrition may depend, at least to some extent, on such valency change. Kenten & Mann (1952*a*) have shown that *in vitro* the oxidation of Mn^{2+} ions can be brought about by enzyme systems producing hydrogen peroxide, such as α-hydroxy-acid oxidase, xanthine oxidase and plant amine oxidase systems, coupled with peroxidase systems. Peroxidase is known to be widely distributed among the higher plants, and Siegel & Galston (1955) and Pilet & Galston (1955) have recently put forward evidence of peroxide genesis in plant tissues. Although the components of the manganese-oxidizing system appear to be widely distributed in the tissues of higher plants there is as yet no evidence that any large part of plant respiration is dependent on this system. Lundegårdh (1954) finds that only a small fraction of the normal aerobic respiration of wheat and rye roots depends on a peroxidase system. It is possible that this fraction of the respiration depends on manganese oxidation. Since peroxidase catalyses the oxidation of Mn^{2+} ions the range of compounds which peroxidase systems can oxidize will be extended by addition of Mn^{2+} ions to include those compounds capable of oxidation by the oxidation product of manganese. Thus Kenten & Mann (1953) have shown that the oxidation of certain dicarboxylic acids is catalysed by peroxidase systems in the presence of Mn^{2+} ions. Although the fraction of the total respiration passing through the peroxidase–manganese system may be small it may be qualitatively important.

SUMMARY

1. Pea plants (*Pisum sativum* L.) grown in water culture containing toxic concentrations of manganese sulphate accumulate compounds containing manganese of valency greater than two in the stems.

2. The presence of these higher-valency forms was demonstrated colorimetrically by the benzidine test, and the amount present estimated spectrophotometrically as manganipyrophosphate and manometrically by the oxidation of hydrazine.

3. It is suggested that the oxidation is due to peroxidase systems. Under toxic conditions of manganese supply the rate of oxidation of Mn^{2+} ions may exceed the rate of reduction of 'higher-valency forms of manganese' by plant metabolites, thus leading to the observed accumulation of manganese higher oxides.

4. In other plants examined under similar conditions the accumulation of 'higher-valency forms of manganese' could not be demonstrated, possibly owing to the fact that in these plants they are reduced as fast as they are formed.

5. It is suggested that the function of manganese as an essential element in plant nutrition depends, to some extent, on such valency change.

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Studies in Immunochemistry

17. THE DECOMPOSITION OF SEROLOGICALLY SPECIFIC H SUBSTANCE ISOLATED FROM PIG GASTRIC MUCIN AND HUMAN OVARIAN CYST FLUIDS BY AN ENZYME PREPARATION OBTAINED FROM *CLOSTRIDIUM WELCHII* (TYPE B)*

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Schiff (1935, 1939) reported that bacteria-free filtrates from cultures of certain strains of *Clostridium welchii* destroyed the serological character of the blood-group A substance in commercial pepsin and human saliva. This finding has been confirmed by many workers. Meyer (1945) observed that an enzyme obtained from *Cl. welchii* liberated free reducing sugars from a neutral mucopolysaccharide which had been isolated from pig gastric mucin (Meyer, Smyth & Palmer, 1937) and which possessed high blood-group A activity. This carbohydrate was later shown to contain two neutral mucopolysaccharides, each of which had similar physical and chemical properties but showed a different serological specificity (Bendich, Kabat & Bezer, 1946; Aminoff, Morgan & Watkins, 1946; Chadwick, Smith, Annison & Morgan, 1949). The digestion of those pig-stomach linings which were free from blood-group A activity gave a neutral mucopolysaccharide which was closely similar in its physical, chemical and serological properties to a mucopolysaccharide found in the water-soluble secretions of the majority of persons belonging to blood group O (Landsteiner & Harte, 1941; Witebsky & Klendshoj, 1941; Morgan & Waddell, 1945). The serological specificity of this material has been designated by the symbol H (Morgan & Watkins, 1948, 1955). The H substance obtained from pig gastric mucosa is of special interest owing to its close similarity to the human H substance and because it can be more easily obtained in a high state of purity and in amounts sufficient for detailed structural studies.

An enzyme which brings about the rapid inactivation of H substances of human and animal origin was obtained from *Cl. welchii* (type B) culture filtrates by Stack & Morgan (1949), who devised a procedure for its purification, described a simple semi-quantitative method for the measurement of enzymic activity and recorded some properties of the enzyme, such as its heat stability, pH of optimum activity, etc. This enzyme preparation was used by Crumpton & Morgan (1953) to inactivate and degrade purified H substance obtained from pig-stomach linings, and it was found that fucose, *N*-acetylglucosamine, galactose and a small amount of a disaccharide composed of *N*-acetylgalactosamine and galactose were liberated from the macromolecule.

Iseki & Tsunoda (1952) obtained from soil an organism which produced an enzyme that specifically inactivated H substance, and Iseki & Masaki (1955) reported that when this enzyme acted on H substance a large part of the fucose contained in it was liberated in a dialysable form but that no other component was set free and no change was induced in the moiety containing amino acid.

Watkins (1955*b*) showed that an enzyme could be extracted from the protozoan flagellate *Trichomonas foetus* which rapidly inactivated H substance and liberated reducing sugars. Part of the material became diffusible through a cellophan membrane and chromatographic examination showed that L-fucose was the main sugar liberated together with a small amount of *N*-acetylhexosamine; galactose appeared to be absent.

The investigations recorded in the present paper are an extension of earlier work (Crumpton & Morgan, 1953) and include some preliminary observations on the enzymic degradation of the H-specific substance of human origin.

METHODS

The determinations of reducing sugars and hexosamines were carried out on materials after hydrolysis with 0·5 N-HCl for 16 hr. at 100°. All other estimations were made on intact materials.

Nitrogen. Nitrogen was determined by the Kjeldahl method with the apparatus of Markham (1942).

Methylpentose. The method of Dische & Shettles (1948), with procedure CyR 10, and later the method as modified by Gibbons (1955), was employed. Fucose standards were included in each determination. Fucose was also determined as acetaldehyde after its liberation from mucopolysaccharide by mild acid and oxidation with periodate in

* Part 16: Gibbons, Morgan & Gibbons (1955).

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Conway units, according to the method of Winnick (1942) as used by Aminoff, Morgan & Watkins (1950).

Hexosamine. A modification (Rondle & Morgan, 1955*a*) of the method of Elson & Morgan (1933) was used. Glucosamine hydrochloride standards in the range 10–50 μg. were included in each determination. Extinctions were read at 530 mμ. with a spectrophotometer. Results are given as hexosamine base.

Reducing sugars. Determined by the method of Somogyi (1937) or by the colorimetric technique of Nelson (1944). Reducing values are expressed throughout as percentage reduction of an equal weight of glucose or as mg. of glucose.

Equivalent N-acetylglucosamine value. This was determined under the conditions recommended by Aminoff, Morgan & Watkins (1952). The results are expressed as a percentage of the colour given by an equal weight of *N*-acetylglucosamine.

Physical examinations. Ultracentrifugal and electrophoretic examinations were carried out as described by Kekwick (1950).

Enzyme activity. Measured as described by Stack & Morgan (1949). The unit of enzymic activity is that amount of activity which will bring about an extent of inactivation of 1 mg. of H substance in 2 hr. at 37° and pH 7 which results in the destruction of 94% of its serological activity. In practice this consists of bringing about a lowering of the inhibiting titre by four tubes when the usual twofold geometrical-dilution scale is employed.

Separation of glucosamine and galactosamine. According to the method of Gardell (1953) as described by Rondle & Morgan (1955*b*), with a column filling of Zeo-Karb 225 and 0·34 N-HCl as eluent.

Chromatography. Chromatograms were run on Whatman no. 1 or 4 paper with (*a*) *n*-butanol–acetic acid–water (4:1:5, by vol.), or (*b*) *n*-butanol–pyridine–water (6:4:3, by vol.), or (*c*) water-saturated *S*-collidine, both the descending and ascending methods being used. In certain instances, in order to obtain a better separation of the components, the papers were dried and run a second time in the same solvent system. Aniline hydrogen phthalate (Partridge, 1949), *p*-anisidine–HCl (Hough, Jones & Wadman, 1950) and benzidine–acetic acid (Horrocks, 1949) sprays were used to detect reducing sugars; ninhydrin was used for amino acids and amino sugars, and alkaline acetylacetone followed by the *p*-dimethylaminobenzaldehyde–HCl reagent as a more specific test for hexosamines (Partridge, 1948). The method of Gardell, Heijkenskjöld & Roch-Norlund (1950), as modified for paper chromatography by Stoffyn & Jeanloz (1954), was used to distinguish glucosamine from galactosamine.

Serological activity. The H activity of the original materials and of the enzymic- and acid-hydrolysis products was determined by the method of inhibition of haemagglutination with (*a*) an immune rabbit anti-H serum (Morgan & Waddell, 1945), (*b*) the human anti-H serum 'Warboys' (Watkins, 1952), and (*c*) the human serum 'Tomlinson' (Watkins & Morgan, 1954). These sera reacted preferentially with group O cells and were completely inhibited by extremely small amounts of H substance. The pneumococcus type XIV activity of the different materials was measured by precipitation-inhibition tests as described by Kabat, Baer, Bezer & Knaub (1948).

MATERIALS

Pig H substance. The preparations of H substance were obtained from pig gastric mucosa according to the method of Bendich *et al.* (1946). The crude mucin obtained from several stomachs, each of which possessed H activity only, was purified by extraction with 90% (w/v) phenol (Morgan & King, 1943) and precipitated from solution in phenol by addition of ethanol to a concentration not exceeding 10% (v/v). The mucopolysaccharide was fractionated further either from solution in 90% (w/v) phenol with ethanol or from aqueous solution with ethanol, or from both. The material precipitating from aqueous solution between 50 and 53% (v/v) ethanol concentrations was most active serologically; it was dissolved in water, dialysed and dried from the frozen state.

Three preparations made from batches of stomach linings were used in the main experiments. The analytical figures for the H materials are given in Table 1. The values given are uncorrected for ash, which was less than 2% of the weight of H substance. Preparations nos. 2 and 3 gave single, symmetrical boundaries in U-tube electrophoresis at pH 4·0 (acetate buffer *I* 0·1) and at pH 8·0 (phosphate buffer *I* 0·2). Examination at pH 8·0 in a Svedberg ultracentrifuge revealed in both preparations a single major component and a small amount of a second substance which sedimented slightly faster. The ultraviolet-absorption spectrum showed only a weak general absorption between 260 and 310 mμ. A fractional-solubility test carried out as described by Aminoff *et al.* (1950), with water as solvent, failed to reveal any gross inhomogeneity.

Human H substance. Isolated from a human ovarian-cyst fluid (no. 247), as described by Morgan & Waddell (1945) and Annison & Morgan (1952). The analytical figures are given in Table 1.

Enzyme preparations. The enzyme was isolated from *Cl. welchii* (type B) culture filtrates (strains CN 1253 and CN 1990, National Type Culture Collection) grown in peptone medium (3% of Evans peptone and 2·5% of sodium β-glycerophosphate) freshly supplemented with glucose and thioglycollic acid as described by Stack & Morgan (1949). The enzyme was recovered by precipitation of the culture filtrate with an equal volume of acetone and purified by reprecipitation from aqueous solution between 25 and 31%

Table 1. *Analytical figures of H substances*

Preparation	N (%)	Fucose (%)	Hexosamine* (%)	Reduction* (%)	Equivalent *N*-acetylglucosamine (%)
H substance no. 1	5·5	9·8	27	49	10·5
H substance no. 2	6·0	10·6	32	51	12·8
H substance no. 3	5·9	10·0	29	50	12·6
Human H substance	5·5	9·8	27	49	4·5

* After hydrolysis with 0·5 N-HCl for 16 hr. at 100°.

saturation of $(NH_4)_2SO_4$, or up to 40% (v/v) of ethanol. Fractionation with $(NH_4)_2SO_4$ was carried out at about 2°, and with ethanol and acetone at about −10°. The selected fractions were finally dialysed against water and dried from the frozen state. All enzyme experiments were made in the presence of McIlvaine buffer (McIlvaine, 1921) at pH 6·8–6·9.

Morgan (1946), Stack & Morgan (1949) and Crumpton (1953) showed that the enzyme which inactivated the H substance was stable on heating in buffer at pH 6·8 for 10 min. at 55°, whereas many of the accompanying enzymes were destroyed. To reduce the number of enzymes present which were not directly involved in the destruction of the serological activity, all the enzyme preparations used in the experiments to be described were heated at 55° for 10 min. The analytical figures for enzyme preparation no. 1 were: N, 8·5%; reducing power, 1·1 and 16% before and after acid hydrolysis respectively; hexosamine about 2% and fucose 0·5%. These values were typical of the other enzyme preparations examined, the N values of which were between 8 and 12%; the reducing values after acid hydrolysis were usually low and the fucose and hexosamine content was less than 2%.

EXPERIMENTS AND RESULTS

Decomposition of H substance by Clostridium welchii *enzyme*

Expt. 1. H substance (prep. 1, 896 mg.) was dissolved in 80 ml. of water saturated with toluene and incubated for 7 hr. at 37° with 10 ml. (about 4000 units) of 1% (w/v) solution of enzyme (prep. 1) in buffer. After incubation less than 5% of the original H activity, measured by immune-rabbit anti-H serum, remained. The mixture of inactive H substance and enzyme was quantitatively transferred to a thoroughly washed cellophan bag (Cellophane sheet no. 300 supplied by British Cellophane Ltd.) and dialysed for a week at 2° against daily changes of water to separate the hydrolysis products into diffusible and indiffusible materials. The diffusates were stored with added toluene at 2° until dialysis was complete, concentrated under reduced pressure at about 15° and made up to a known volume. The indiffusible material was recovered by drying the contents of the cellophan bag from the frozen state.

The total nitrogen, fucose, hexosamine and reducing sugar in the diffusible and indiffusible hydrolysis products were determined.

In order to interpret quantitatively the results it is necessary to know how much of the added enzyme preparation was diffusible and the composition of the diffusible and indiffusible material. As a control in Expt. 1 the enzyme [10 ml. of 1% (w/v) solution in buffer] was therefore dialysed after it had been incubated for 7 hr. in the same final volume (90 ml.) as that used in the experiment containing H substance. This treatment was considered to simulate as closely as possible the conditions to which the enzyme preparation was exposed during incubation with the substrate. The analytical figures for the diffusible and indiffusible part of the enzyme were determined as in the main experiment.

Expt. 2. The procedure used was the same as given in Expt. 1 above. H substance (prep. no. 2, 444 mg.) dissolved in 39 ml. of water was incubated for 7 hr. at 37° with 5 ml. (about 590 units, 4·4 mg. of nitrogen) of 1% (w/v) solution of enzyme (prep. no. 2) in buffer. After incubation not more than 3% of the original serological activity remained. The products were separated into diffusible and indiffusible materials and analysed as described in Expt. 1. The enzyme control (50 mg. of enzyme in 44 ml. of dilute buffer, incubated for 7 hr. at 37°) was similarly treated. In Expt. 2, however, an additional or 'substrate' control was included. H substance (90 mg. in 8 ml. of water) was incubated for 7 hr. with 4 mg. of enzyme which had previously been heated in 1 ml. of buffer at 100° for 10 min. The substrate was recovered after dialysis and the analytical composition found to be close to that of the original material. The slight differences found between the recovered material (nitrogen, 6·1; fucose, 9·7; hexosamine, 30%; reduction, 50%), which was serologically fully active, and the original H substance (prep. no. 2) were most probably due to the presence of some enzyme material.

Expt. 3. H substance (prep. no. 3, 862 mg.) dissolved in 83 ml. of water and incubated for 7 hr. with 78 mg. (2200 H units, 8·7 mg. of nitrogen) of enzyme (prep. no. 3) in 10 ml. of buffer. The specific serological activity of the substrate measured against the rabbit anti-H serum was completely destroyed. The products of hydrolysis were recovered and analysed as were the corresponding fractions obtained from the enzyme control.

Expt. 4. Human H substance (779 mg.) in 80 ml. of water was incubated with 4 ml. of 1% (w/v) enzyme solution (about 1600 units). After incubation for 4 hr. a further 4 ml. of enzyme solution was added and incubation continued for an additional 3 hr. The products of hydrolysis were separated into diffusible and indiffusible fractions and analysed. An enzyme control experiment was included. The results are given in Table 4.

Examination of the products of enzymic hydrolysis

Reliable weights for the diffusible substances, which were recovered as hygroscopic sugar syrups, were not obtained; an approximate estimate of their amount was given by deducting the weight of the indiffusible substances, corrected for enzyme content, from the weight of the original substrate. The results showed that one-fifth to one-third of the substrate became diffusible in different experiments, a variation which is presumably due largely to the

enzyme preparation employed. Further decomposition then ceased, although the enzyme, after dialysis and removal of certain of the hydrolysis products, was found to be active when tested on fresh substrate. In each of the four main experiments described not less than 95% of the original serological activity was destroyed.

Physical properties. The immediate action of the enzyme preparation on solutions of the H substance was to bring about a rapid fall in their viscosity. The H substance in Expt. 2, for example, showed a relative viscosity of 26 when measured in an Ostwald-type viscometer at 1% (w/v) concentration in 0·85% saline at 37°, whereas the indiffusible material remaining after the action of the enzyme and accounting for about three-quarters of the original substrate gave a value close to 5 (saline 1). In Expts. 2 and 3 the indiffusible residues (1%, w/v) were examined in the ultracentrifuge at pH 8 (sodium phosphate buffer, I 0·2, 0·15M-NaCl). Apart from a small quantity of rapidly sedimenting substance, which was visible in the early schlieren diagrams only and was most probably enzyme protein, the materials gave sharp boundaries, sedimented at a slightly slower rate than did the original H substances and behaved as might be expected for a single substance. The indiffusible material obtained from Expt. 3 was examined electrophoretically at pH 8 and was found to contain one major (about 90%) and at least one minor component. The ultraviolet-absorption spectrum of the product was similar to that of the original H substance.

Chemical properties. The analytical values obtained for the original substrates and for the diffusible and indiffusible products in Expts. 1–3 are given in Table 2. The values shown are corrected for the contribution made by the enzyme to the particular material estimated. The corrections necessary in each instance are relatively small (Table 3).

The figures given (Table 2) show that fucose was preferentially liberated, for whereas in Expts. 1–3 about 31, 25 and 29% of the substrate became diffusible, 37, 57 and 71% of the total fucose respectively was liberated in each instance.

Table 2. *Amounts and distribution of nitrogen, fucose, hexosamine and reducing sugars in the enzymic hydrolysis products of H substance*

	N			Fucose			Hexosamine*			Reducing sugar*		
Expt. no.	1	2	3	1	2	3	1	2	3	1	2	3
Original H substance (mg.)	49	27	51	88	47	86	245	140	247	435	227	433
Indiffusible material (mg.)	39	25	47	61	23	25	179	96	144	339	157	256
Diffusible material (mg.)	9	3	7	32	27	61	53	38	80	98	78	158
Components accounted for (%)	96	103	106	106	106	100	95	96	91	101	103	96
Amount diffusible (%)	18	11	14	37	57	71	22	27	32	23	34	36

* After hydrolysis with 0·5N-HCl for 16 hr. at 100°.

Table 3. *Values of the corrections applied to the enzymic hydrolysis products for the enzyme material present in (a) the diffusible material and (b) the indiffusible residue*

Expt. no.	Nitrogen (mg.)		Fucose (mg.)		Hexosamine* (mg.)		Reduction* (mg.)	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
1	2·0	5·3	<1	<1	<1	1·2	3	13
2	0·4	2·4	<1	<1	<1	<1	<1	<1
3	2·2	4·9	<1	<1	<1	1·1	4	9
4	2	5·5	<1	<1	<1	1·5	3·3	16

* After hydrolysis with 0·5N-HCl for 16 hr. at 100°.

Table 4. *Distribution of components after treatment of human H substance with* Clostridium welchii *enzyme*

Component	H substance (mg.)	Indiffusible material (mg.)	Diffusible material (mg.)	Amount of component accounted for (%)	Component diffusible (% of total)
Nitrogen	59	39	5·8	76	10
Reduction	9	27	60	—	—
Reduction*	372	232	81	84	22
Fucose	114	65	29	83	26
Hexosamine*	160	115	38	96	24

* After hydrolysis with 0·5N-HCl for 16 hr. at 100°.

Concomitant with the release of diffusible material during enzymic hydrolysis of H substance there was a rapid formation of reducing groups in both the diffusible and indiffusible products. The H substance used in Expt. 1 (896 mg.) gave a reducing value equivalent to 9 mg. of glucose or about 1% reduction, whereas the indiffusible residue (616 mg.) showed a reducing power equal to 33 mg. of glucose or nearly 5·4% reduction. The diffusible material contained a much larger proportion of reducing groups, equivalent in reduction to 59 mg. of glucose, or about 21% on a weight basis. If the whole of the fucose (32 mg.) in the diffusible material had been present as free sugar, the reduction expected would correspond to about 15 mg. of glucose, as fucose has a reducing power equivalent to about 45% of its weight of glucose. It is evident, therefore, that a part of the reducing power of the diffusate arises from sugars other than fucose. Similar results were obtained in Expts. 2 and 3, where the diffusible materials gave a reducing power equivalent to 63 and 113 mg. of glucose respectively, or 57 and 45% reduction of the weights (111 and 251 mg.) obtained by subtracting the weights of the indiffusible materials, corrected for enzyme content, from the weights of H substances used.

In Expt. 1 (Table 2), of the 245 mg. of total hexosamine in the original substrate 179 mg. remained in the indiffusible residue, and the diffusible material contained 53 mg.; thus 95% of the total hexosamine was accounted for. The ratio of glucosamine to galactosamine in the H substance used was 3·2:1, whereas in the diffusate the ratio was found to be 5·5:1; a preferential release of glucosamine had occurred during the early stages of hydrolysis.

Amino acids. The amino acids contained in the H substance were not liberated to any appreciable extent in a diffusible form during the early stages of enzymic hydrolysis. The analytical figures on which this statement is made, however, are based on the difference between the total nitrogen and the total hexosamine nitrogen, and not on a direct determination of amino acid nitrogen. A part of the small amount of amino acids found in the diffusates undoubtedly comes from autolysis of protein in the enzyme preparation.

Action of mild acid. The rate of liberation of fucose from H substance and from the indiffusible residue obtained in Expt. 2 during hydrolysis with N acetic acid at 100° is shown in Fig. 1. The liberation of fucose from H substance ceased after about 36 hr. and the indiffusible material gave no further diffusible fucose after hydrolysis for about 18 hr. The maximum amounts of fucose set free were 8·6 and 4·2% respectively of the materials used. The total fucose content of the H substance and of the indiffusible material was 10·6 and 6·8% respectively and therefore these two materials contained 2·0 and 2·6% of 'acid-stable' fucose. The original H substance contained 8·9 mg. (2·0% of 444 mg.) of fucose resistant to hydrolysis with mild acid, whereas the indiffusible residue (333 mg.) obtained from 444 mg. of H substance (Expt. 2) contained 8·7 mg. (2·6% of 333 mg.) of 'acid-stable' fucose. This result suggests that in the H substance there are fucose molecules not readily liberated by the enzyme or by acid hydrolysis.

Action of alkali. It has been long known that treatment of the H substance with dilute alkali under carefully controlled conditions gives a chromogenic structure which forms a coloured complex with *p*-dimethylaminobenzaldehyde–HCl reagent (Morgan & King, 1943; Morgan, 1947; Morgan & Waddell, 1945; Annison & Morgan, 1952), and that the formation of the chromogen depends on the presence of *N*-acetylhexosamine containing a reducing end-group (Aminoff *et al.* 1952) and on the presence of an unsubstituted OH group attached to C-4 (Kuhn, Gauhe & Baer, 1954*b*). A preformed chromogenic structure was present neither in the H substance nor in the diffusible or indiffusible materials derived from it by enzymic hydrolysis. However, the products of enzymic hydrolysis gave a chromogenic structure on treatment with dilute alkali which showed an absorption spectrum closely similar to that given by H substance or *N*-acetylglucosamine after similar treatment.

The maximum colour intensity obtained with known weights of these materials can be expressed

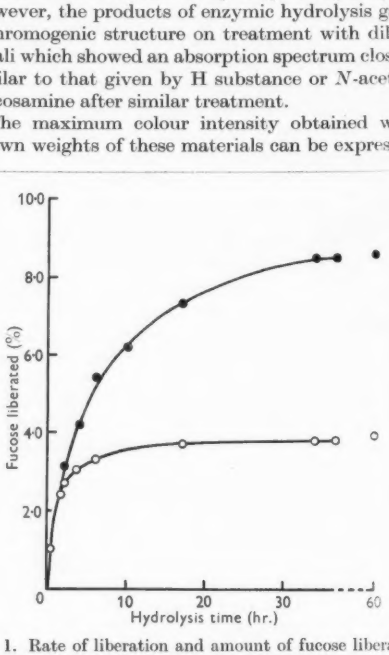

Fig. 1. Rate of liberation and amount of fucose liberated by mild acid hydrolysis from H substance (prep. no. 2; ●) and from the indiffusible material (○) formed by treatment with *Cl. welchii* enzyme preparation.

in terms of the maximum colour intensity developed under closely defined conditions by *N*-acetylglucosamine (see Aminoff *et al.* 1952). It is necessary, however, to determine for each product the optimum time of heating at 100° with alkali (0·05 N-Na_2CO_3) which subsequently gives the maximum colour intensities at 550 mμ. and 590 mμ. on the addition of the *p*-aminodimethylbenzaldehyde–HCl reagent. For *N*-acetylglucosamine and *N*-acetylgalactosamine the optimum heating period under the conditions employed was 4 min. The undegraded H substance required 8–12 min. heating with alkali, the indiffusible materials 6–8 min., and the diffusible products 3–4 min., the times varying somewhat with the products obtained in different experiments.

The maximum colour intensities determined under these conditions for the materials obtained in Expts. 1–3, and expressed as mg. of *N*-acetylglucosamine and as per cent of the weight of the fractions, are given in Table 5. It is known that there is some destruction of the chromogen under the conditions selected for its formation, that free *N*-acetylgalactosamine gives only about one-fifth of the colour given by an equal weight of *N*-acetylglucosamine (Aminoff *et al.* 1952), that *N*-acetylglucosamine substituted in the fourth hydroxyl group gives no colour (Kuhn *et al.* 1954*b*) and that certain oligosaccharide structures give more colour than their content of hexosamine would lead one to expect (Kuhn, Gauhe & Baer, 1954*a*). For these reasons, the full significance of the final equivalent *N*-acetylglucosamine values obtained for each material cannot be fully appreciated; nevertheless, the figures are of considerable value for comparative purposes and show that the indiffusible products give a similar amount of '*N*-acetylhexosamine' colour to that found for the original H substance, whereas the diffusible materials give a colour equivalent to up to one-half their weight of *N*-acetylglucosamine.

The diffusible materials, on treatment with 0·05 N-Na_2CO_3 at room temperature, formed a certain amount of chromogen, whereas after similar treatment *N*-acetylglucosamine and *N*-acetylgalactosamine failed to undergo this change. A substance reactive in alkali under these conditions was isolated by paper chromatography (see below), but was not completely identified. In view of the fact that *O*-β-D-galactopyranosyl-(1 → 3)-*N*-acetyl-D-glucosamine, an alkali-sensitive disaccharide, has been isolated from group A substance (Côté & Morgan, unpublished work) it seems probable that a similar structure is present in H substance and is responsible for the sensitivity to alkali and the ease of chromogen formation. The substance gave a chromogenic structure on treatment in aqueous $BaCO_3$ suspension (pH 8·4) for 15 min. at 100° (cf. Knox, 1954; Knox & Morgan, 1954), as did the *O*-β-D-galactopyranosyl-(1 → 3)-*N*-acetyl-D-glucosamine. Under these extremely mild conditions *N*-acetylglucosamine and *N*-acetylgalactosamine require about 4 hr. and H substance 48 hr. to give maximum chromogen formation.

The results obtained in Expt. 4, in which the products of enzyme hydrolysis of the human H substance were treated with alkali to determine the ease and extent of chromogen formation (Aminoff *et al.* 1952), are given in Table 5; the materials behaved as did those obtained from the pig H substance.

Serological properties. The H substances and their indiffusible and diffusible enzymic hydrolysis products were examined serologically with different anti-H sera. Tested for activity against rabbit-immune H serum and the human anti-H serum 'Tomlinson' the indiffusible materials from Expts. 1, 2 and 4 showed only a few per cent of the activity of the original H substance, whereas the products obtained in Expt. 3 possessed no demonstrable activity. However, the capacity of these materials to react with the anti-H sera 'Warboys' was unimpaired.

The indiffusible material obtained from Expt. 2, when tested at 0·1 % (w/v) concentration, failed to neutralize the following agglutinins: human anti-A with A_1 or A_2 test-cells; natural human and immune-rabbit anti-B, anti-Le^a, anti-Le^b, anti-M, anti-N, anti-P and anti-*Rhesus* sera (including anti-D, anti-C, anti-c and anti-E test reagents). The diffusible material was inactive with all the test antisera.

Table 5. *Equivalent* N-*acetylglucosamine value of the H substance and of the enzymic hydrolysis products after treatment with alkali and the addition of the* p-*dimethylaminobenzaldehyde*–HCl *reagent*

Maximum colour intensity obtained expressed as (*a*) mg. of *N*-acetylglucosamine and (*b*) as % of weight of fraction.

Expt. no.	H substance		Indiffusible material		Diffusible material	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
1	93	10	78	11	72	25
2	57	13	44	13	50	45
3	109	13	74	12	104	41
4	34	4	44	7	45	19

The capacity of the H substance and of the indiffusible enzymic hydrolysis products to react with the anti-H serum 'Warboys' was readily destroyed on treatment with N acetic acid at 100°.

The enzymic hydrolysis products obtained from H substance failed to precipitate with horse anti-pneumococcus type XIV serum. The products obtained from the H substance after hydrolysis with acetic acid for 2 hr., however, reacted strongly with this serum, but the products lost this property on treatment with the *Cl. welchii* enzyme. The type XIV serum failed to precipitate with its homologous specific polysaccharide after this material had been treated with the enzyme.

Chromatographic analysis. The hydrolysate obtained by treating the diffusible materials with 0·5N-HCl for 16 hr. at 100° was subjected to paper chromatography in some or all of the solvent systems given under Methods. It gave spots whose R_F values and colours were identical with those of fucose, galactose and the two hexosamines run at the same time; no other sugars were detected.

The unhydrolysed diffusible materials gave two strong spots and one weak spot with aniline hydrogen phthalate which agreed in position with those found for fucose, galactose and *N*-acetylglucosamine run in *s*-collidine at the same time. Two reddish purple spots appeared after spraying with the hexosamine reagents. One of these occupied a position identical with that found for *N*-acetylglucosamine (R_F 0·50); *N*-acetylgalactosamine moves at about the same speed in *s*-collidine. The other spot, R_F about 0·25 in *s*-collidine, could not be identified with any of the simple sugars included for comparison. The material responsible for this unidentified spot was characterized as follows: unhydrolysed diffusate was taken to dryness, reconstituted in 1 ml. of water and examined by the usual method of band chromatography with marginal vertical strips to detect the position of the different components. The chromatograms were developed in *s*-collidine for 40 hr. The eluate containing the material (R_F 0·25) was taken to dryness, the residue dissolved in 1·5 ml. of water and 1 ml. of this solution was hydrolysed at 100° in 0·5N-HCl for 16 hr. The hydrolysed and unhydrolysed eluates were then re-examined chromatographically with *s*-collidine as solvent. The unhydrolysed eluate gave a weak or negative reaction with aniline hydrogen phthalate, but after acid hydrolysis gave a strongly positive reaction; the position of the spot with this reagent now corresponded with that of galactose run at the same time. The unhydrolysed eluate gave with the hexosamine reagents a reddish purple colour and possessed an R_F value identical with that found for the unidentified sugar in the original diffusate. The eluate after hydrolysis with 0·5N-HCl at 100° for 16 hr. and running in *s*-collidine gave two spots with the hexosamine reagents which corresponded in position and colour (cherry-red) with those expected for glucosamine and galactosamine (see Aminoff & Morgan, 1948). The galactosamine spot was much less intense than that due to glucosamine. These results suggested that the unidentified material could be a mixture of two (or more) disaccharides composed of *N*-acetylglucosamine or *N*-acetylgalactosamine joined to galactose. The presence of a trisaccharide was not excluded but it seemed improbable that higher oligosaccharides were involved.

The results obtained by spraying the chromatograms of the diffusible material with ninhydrin, aniline hydrogen phthalate and hexosamine reagents indicated that probably a relatively small amount only of the total diffusate remained on or close to the base line in the paper chromatograms.

Chromatograms of the indiffusible materials, which had been hydrolysed at 100° with 0·5N-HCl for 16 hr., or with 6N-HCl for 2 or 4 hr., revealed the presence of fucose, galactose, glucosamine and galactosamine and the same amino acid mixture as was present in the original H substance.

Repeated treatment with enzyme

Pig H substance. Treatment of the indiffusible residue obtained in Expt. 1 with a further amount of enzyme showed that some additional hydrolysis occurred. The analytical figures obtained for the diffusible and indiffusible materials are given in Table 6. About one-third of the substrate became diffusible and this material after treatment with dilute alkali and *p*-dimethylaminobenzaldehyde reagent gave a colour equivalent to 27 mg. of *N*-acetylglucosamine. Chromatographic examination of the diffusate showed that oligosaccharide units larger than disaccharides were probably not present to any appreciable extent. The reducing power of the diffusible material, equivalent to 33 mg. of glucose, indicated that a considerable part of the carbohydrate consisted of small reducing sugar units.

The indiffusible residue was recovered and treated again with enzyme. About 15% of the substrate became diffusible and this material contained 3·7 mg. of nitrogen or about 14% of the original total nitrogen (26 mg.). A much smaller amount (3 mg.) of the total fucose (29 mg.) was released in a diffusible form this time and was accompanied by 10 mg. or about 10% of the total hexosamine. The reducing power of the diffusible material, before and after acid hydrolysis, was equivalent to 11 and 21 mg. of glucose respectively, which indicated again that the major part of the diffusible carbohydrate was made up of small reducing sugar units.

Table 6. *Distribution of components after treatment of the indiffusible material from Expt. 1 with additional* Clostridium welchii *enzyme*

Component	Substrate indiffusible material from Expt. 1 (mg.)	Indiffusible material (mg.)	Diffusible material (mg.)	Amount of component accounted for (%)	Component diffusible (% of total)
Nitrogen	41	28	6·1	85	15
Reduction	31	19	33	—	—
Reduction*	327	221	58	85	18
Fucose	57	34	18	91	32
Hexosamine*	167	111	26	83	16

* After hydrolysis with 0·5 N-HCl for 16 hr. at 100°.

Human H substance. The indiffusible material (397 mg.) obtained in Expt. 4 was treated with a further amount of enzyme (40 mg.) for 7 hr. at 37°. An indiffusible residue (261 mg.) was obtained which contained 15 mg. of nitrogen, 105 mg. of reducing sugar after acid hydrolysis, 48 mg. of hexosamine and 28 mg. of fucose. The diffusible material contained 5·6 mg. of nitrogen, 23 and 47 mg. of reducing substances (as glucose) before and after acid hydrolysis, 12 mg. of fucose and 18 mg. of hexosamine. About one-quarter of the starting material diffused through the cellophan membrane after treatment with enzyme and it is again evident that there was a considerable increase in the number of reducing groups present, especially in the diffusible material.

Action of other enzyme preparations on H substance

The following materials, shown to contain an α-glucosaminidase, failed to destroy the serological activity of H substance in 24 hr. at pH 6·9: crude ox-testis extract; Rondase (hyaluronidase, Evans) and an extract of snail (*Helix aspersa*) digestive tract. Similarly, preparations of β-galactosidase derived from emulsin, snail juice and *Bacterium coli* (strain M.L. 308) did not cause any measurable loss of serological activity of the H substance.

DISCUSSION

An attempt has been made to determine the changes induced in H substances of animal and human origin by an enzyme preparation obtained from culture filtrates of *Cl. welchii* (type B). The animal H preparations were obtained from pig-stomach linings and the human specific substance from an ovarian-cyst fluid. The materials possessed similar physical, chemical and serological properties and showed no gross inhomogeneity when examined by fractional-solubility tests, by electrophoresis or by ultracentrifuging. Each material contained fucose, galactose, glucosamine, galactosamine and eleven amino acids, and inhibited, in extremely small amounts, the agglutination of O cells by certain human and animal anti-H agglutinins. The serological specificity of the materials was almost completely destroyed by 10% (w/v) of the enzyme preparation acting for 7 hr. at pH 6·8–7·0 at 37°.

The action of the enzyme on the H substances caused an immediate and rapid fall in the viscosity and serological activity, and liberated in a diffusible form a part of the fucose, galactose and *N*-acetylhexosamine present. After hydrolysis sufficient to bring about almost complete serological inactivation of the substrate, measured against a rabbit-immune anti-H serum, the products were in each instance separated into materials which were diffusible or indiffusible through a cellophan membrane. The extent to which the products were contaminated by the enzyme preparation was determined from control experiments and a correction made to the analytical figures obtained.

The distribution of the various components of the macromolecule in the diffusible material was found to be very different from that which might be expected if each sugar and amino acid residue had been liberated to a similar extent in a diffusible form and it was immediately apparent that there was a preferential release of fucose.

Chromatographic examination of the diffusates indicated that a considerable part of these materials consisted of single sugar residues together with small amounts of di- and possibly higher saccharides. This conclusion is supported by the high reducing power found for the diffusates which contain much fucose, a sugar which gives a reduction equivalent to rather less than one-half of its weight of glucose. Although it is possible that oligosaccharide units greater than disaccharides exist in the diffusates to a small extent, it is now known from the results of partial hydrolysis of the group substances with mild acid that oligosaccharides which contain fucose and *N*-acetylhexosamine residues move relatively fast on paper chromatograms in the solvents used, and might in consequence appear in a position normally occupied by smaller saccharide units. Until a full identification of the sugar units liberated has been made it is not possible to state with any certainty to

what extent oligosaccharides greater than disaccharides occur in the diffusible materials.

Some of the analytical figures (expressed in mg.) which were obtained for the diffusible and indiffusible hydrolysis products are expressed as ratios in Table 7. The values for the ratio of amounts of reducing sugar to nitrogen, reducing sugar to non-hexosamine nitrogen and glucosamine to galactosamine for the diffusible material show a considerable increase over similar ratios obtained for the unchanged substrate. The ratio of reducing sugar to fucose, however, is much reduced. These findings indicate that there is a preferential liberation of fucose and glucosamine in the early stages of the hydrolysis.

The original H substance gave a reduction equivalent to less than 2% of its weight of glucose, whereas the corresponding reduction given by the indiffusible material was 5%. If it is assumed that the indiffusible residue is essentially a single molecular species, as appears possible from the limited physical measurements made, then 5% reduction would suggest that the residue contains straight chains each made up of about 20 sugar units. Such a unit-chain structure would possess a minimum molecular weight of about 3000, or rather more if the reducing end-unit in the chain was fucose or *N*-acetylhexosamine.

A part of the *N*-acetylhexosamine liberated from the H substance was in a form which was readily diffusible and reactive in dilute alkali to give a chromogenic structure. It was possible by paper chromatography to isolate from the diffusates a small amount of material—most probably of disaccharide nature—which formed chromogen under alkaline conditions unable to bring about chromogen formation with *N*-acetylglucosamine or *N*-acetylgalactosamine. Kuhn *et al.* (1954*b*) obtained from a tetrasaccharide present in human milk a disaccharide which reacted more readily with alkali than did *N*-acetylglucosamine, and Kuhn, Baer & Gauhe (1954) subsequently identified the alkali-labile material as 3-β-galactosyl-*N*-acetylglucosamine. This alkali-sensitive disaccharide has also been isolated from the partial acid hydrolysis products of group A substance (unpublished results).

An attempt has been made to determine the changes which occur when the indiffusible material resulting from the first treatment of H substance with enzyme is recovered and treated again with the enzyme preparation. An indiffusible material remained which seemed to be increasingly resistant to further breakdown by the enzyme preparation. The qualitative composition of the indiffusible residue was similar to the original H substance; but the extent of the quantitative changes were more difficult to determine as the analytical figures are not entirely reliable, owing to the steadily increasing contamination of the indiffusible residues with the unspecific material which accompanies the enzyme. For this reason the results are considered provisional. However, the ratio of the amount of hexosamine to fucose, two substances which occur in small amounts only in the enzyme preparations, changed steadily from 2·8 in one preparation of H substance to 4·4 in the indiffusible material obtained after three treatments with the enzyme preparation. The corresponding ratio found for the indiffusible residues obtained from the human H substance likewise showed the same upward trend.

The enzyme preparation liberated up to one-third of the *N*-acetylhexosamine units from the H substances (Expt. 3), yet the resulting indiffusible residue showed about the same percentage of '*N*-acetylhexosamine' colour (see Table 5) as did the original H substance. It seems probable, therefore, that the structure in the H substance which gives this colour cannot be solely responsible for its serological reactivity with anti-H serum.

The serological properties of the H substance, measured by rabbit anti-H serum or the human serum 'Tomlinson', were rapidly destroyed by the enzyme preparation but the hydrolysis products still reacted strongly with the serum 'Warboys'. The capacity to react with this reagent was confined to the indiffusible material.

Treatment of the H substance with the enzyme caused a considerable part of the fucose, together

Table 7. *Ratios of the analytical values (mg.) obtained for the H substance and the indiffusible and diffusible materials after treatment with enzyme in Expt.* 1

Ratio	Original H substance	Materials obtained after enzyme treatment	
		Indiffusible	Diffusible
Reducing sugar:N	8·9	8·7	10·9
Reducing sugar:non-hexosamine N	14·6	12·6	22·0
Reducing sugar:hexosamine	1·8	1·9	1·9
Reducing sugar:fucose	4·9	5·6	3·0
Glucosamine:galactosamine	3·2	2·9	5·5
Hexosamine:fucose	2·8	2·9	1·6
Non-hexosamine N:hexosamine N	1·6	2·3	1·1

with some *N*-acetylhexosamine and galactose, to be released, and it is known that treatment of the H substance with N acetic acid at 100° likewise sets free, in a diffusible form, the major part of the total fucose and destroys the capacity of the material to react with all the anti-H reagents tested, including the serum 'Warboys'. The serologically reactive structure detected by the serum 'Warboys' is therefore different from that reactive with the other anti-H reagents and presumably does not depend on the structural contribution made by the sugars liberated by the enzyme. It appears that some acid-labile structure other than that associated with the sugars liberated by enzymic hydrolysis is essential and must remain intact for reactivity with the serum 'Warboys'. Presumably the 'Warboys' reactive structure is a part only of that responsible for full H specificity. A mucopolysaccharide which reacts with 'Warboys' serum but not with rabbit or human anti-H sera has been encountered in cyst fluids (cf. substance 126/1, Table 1, Gibbons, Morgan & Gibbons, 1955). This material, however, has a fucose content of about 25%.

At the same time as the indiffusible material lost its reactivity with the serum 'Warboys' during hydrolysis with acetic acid, there developed the property of forming a precipitate with horse anti-pneumococcus type XIV serum. This new serological activity was, however, always less pronounced than was the same activity developed by heating the original H substance with mild acid. Kabat *et al.* (1948) suggested that the reactivity of the blood-group substances with type XIV serum after acid hydrolysis was due to the exposure of an *N*-acetylglucosamine–galactose structure similar to that present in the pneumococcus type XIV polysaccharide. If this suggestion is correct then it seems that such an *N*-acetylglucosamine–galactose type of unit is not exposed in a serologically reactive form during enzymic hydrolysis of H substance, or if exposed is rapidly destroyed, as the products fail to react with the anti-pneumococcus serum.

Watkins & Morgan (1955) showed that L-fucose inhibits the enzyme in *Cl. welchii* culture filtrates, which brings about the serological inactivation of H substance, and concluded that L-fucose is an important part of the chemical structure which is responsible for H specificity. The methyl fucopyranosides and furanosides (Watkins, 1955*a*) fail to inhibit the inactivation of H substance by *Cl. welchii* enzyme, but it is known that the similar H-inactivating enzyme in extracts of *Trichomonas foetus* has no action on these fucose derivatives. The presence of a fucosidase in the *Cl. welchii* enzyme preparation, however, must be assumed because of the rapid liberation of free fucose when the enzyme acts on H substance. However, nothing definite is known as to the influence of the nature of the aglycon on the activity of fucosidases, but it seems probable that if the aglycon had been a carbohydrate residue the glycoside bonds would have been readily split. In the light of the earlier observation that the H agglutinins in eel serum (Watkins & Morgan, 1952) and those in an aqueous extract of the seeds of *Lotus tetragonolobus* (Morgan & Watkins, 1953) are inhibited in their action on group O cells by L-fucose, and even more strongly by the glycoside α-methyl-L-fucopyranoside, but by no other simple sugar component of the H substance, it seems probable that an L-fucose end-group plays an important part in the structure responsible for H activity.

The presence of many enzymes in the partially purified *Cl. welchii* enzyme preparation used throughout the present work complicates the interpretation of the results, and for this reason it would be unwise to draw any firm conclusions from the results obtained concerning the structure of the H substance. Since most of the work described in this paper was completed much progress has been made in the separation of mixed enzyme preparations by electrophoresis on starch and cellulose columns, and it is already evident that the application of this and other techniques to the purification of the *Cl. welchii* enzyme preparation is an indispensable step in studies of this kind. Further purification of *Cl. welchii* enzyme, and the discovery (Watkins, 1953, 1955*b*) of a potent source of H enzyme in the protozoan flagellate *T. foetus*, will allow the enzymic approach to the problem of mucopolysaccharide structure to be extended.

SUMMARY

1. Some changes brought about by the action of an enzyme preparation obtained from *Clostridium welchii* (type B) culture filtrates on the serologically specific H substances of animal and human origin are described.

2. When H character, determined by the inhibition of the agglutination of group O cells by immune-rabbit anti-H sera, is almost completely destroyed about one-quarter of the substrate is diffusible through a cellophan membrane.

3. The diffusible material contains the major part of the total fucose, some *N*-acetylglucosamine and galactose and small amounts of a material, possibly a disaccharide, and amino acid-containing residues. The diffusates contain a carbohydrate material which, in dilute alkali at room temperature, forms a chromogen which reacts with *p*-dimethylaminobenzaldehyde.

4. The indiffusible material probably consists largely of a single molecular species and, although containing considerably less fucose and a smaller amount of *N*-acetylhexosamine and galactose, is qualitatively similar to the original H substance.

5. Repeated treatment of the H substance with the enzyme fails to render it completely diffusible through cellophan.

6. The indiffusible residue, which has no capacity to inhibit the agglutination of O cells by immune-rabbit anti-H serum or by the human serum 'Tomlinson', is nevertheless fully active when tested with the anti-H serum 'Warboys'.

7. The capacity of the H substance to react with the anti-H serum 'Warboys' does not depend on the presence of acid-labile fucose within the mucopolysaccharide but probably does depend on some other, equally acid-labile structure.

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The Preparation of ^{32}P-Labelled Adenosine Polyphosphates

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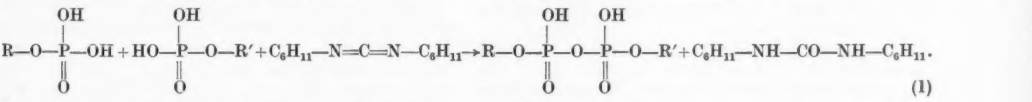
This paper describes the preparation by chemical methods of adenosine polyphosphates† labelled with radioactive phosphorus.

Several biochemical methods of preparing P*-labelled adenine nucleotides with specific activities which are of practical use have been described. The method of Hems & Bartley (1953) for preparing APP*P* utilizes respiring sheep-heart suspensions as the phosphorylating agent. The specific activity of the product is relatively high, but the yield in terms of P* incorporated is low. A similar method, which involves the use of rat-liver mitochondria, has been described by Derache & Lowy (1955), and another method, which involves the use of purified enzyme systems, has been described by Rosenberg (1955). The method of Eggleston (1954) employs a yeast extract for the preparation of AP*. The specific activity of the product and the yield, in terms of P* incorporated, are high.

A variety of chemical methods of synthesizing nucleoside polyphosphates have been used by Todd (1955) and his co-workers. The method which is described here is based on that introduced by Khorana & Todd (1953) for the preparation of symmetrical di- and tetra-esters of pyrophosphoric acid. It has been used by Khorana (1954) and by Hall & Khorana (1954) for the synthesis of adenosine and uridine polyphosphates. The basis of the method is the reaction of DCC with acids. Equation (1) shows the reaction for monoesters of phosphoric acid.



(1)

When R = R′, the product is a symmetrical diester of pyrophosphoric acid. When R and R′ are different, at least three products are possible. If, however, one of the reactants is unsubstituted orthophosphate (R = –OH) then the building up of a polyphosphate chain becomes possible, e.g. when R = –OH and R′ = adenosyl, the products include APP, APPP and probably higher polyphosphates (Khorana, 1954).

In his preparation of adenosine polyphosphates, Khorana (1954) considered it desirable to use an excess of P over AP in order to minimize the formation of the symmetrical APPA. Accordingly, P and AP were used in a molar ratio of about 15:1. Thus when the object is a high yield of adenosine polyphosphates in terms of AP used as starting material, the use of an excess of phosphoric acid is indicated.

In the preparation of labelled nucleoside polyphosphates, the object becomes one of maximum yield in terms of P*, rather than of yield in terms of AP. Another object is to obtain a product possessing a high specific activity. These considerations suggest that the reaction should be tested with successively smaller ratios of P:AP. The smallest P:AP ratios, as well as the highest specific activity possible, are attained if carrier-free P* is used. (The latter, as supplied by the Radiochemical Centre, Amersham, has a specific activity of about 1 mc/μg. of phosphorus.)

Preliminary tests of the reaction revealed that a condensation of carrier-free P* with AP does indeed take place. Analyses showed the main radioactive product to consist of APP*.

When the starting materials were APP and carrier-free P*, the main radioactive products consisted of APPP* and APP*P*. When the starting materials were APPP and carrier-free P*, the main product was radioactive APPPP. The distribution of P* in this product was not studied.

When the starting materials were AP and P*P* the main reaction product was APP*P*.

The identification of the purified products of the reactions described in this paper has depended upon comparisons with reference compounds. The first comparison consists of cochromatography on filter paper. The second comparison is an extension of the principle of cochromatography applied to radio-

† Adenosine mono-, di-, tri-, and tetra-phosphate are represented by AP, APP, APPP and APPPP respectively, and orthophosphate and pyrophosphate by P and PP respectively. Radioactive phosphate is indicated by a suffixed asterisk, thus P*. The phosphorus atoms of the polyphosphate chain are differentiated by the symbols α, β, γ and δ, where α is the position of the phosphorus atom adjoining the sugar. Similar representations have been employed by Knivett (1954) and by Hems & Bartley (1953). Other abbreviations used are APPA, *s*-di(adenosine 5′)-pyrophosphate, and DCC, *NN*′-di*cyclo*hexylcarbodiimide.

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active compounds. The radioactive reaction product and the reference compound are cochromatographed, the latter being present in excess. The shape of the radioactive spot is determined by autoradiography, that of the reference spot by some other means (in the work described here by ultraviolet photography). The compounds can be considered to be identical only if the irregularities in the shapes of the spots recorded by these independent methods of detection coincide accurately. The method has been discussed fully by Roberts, Abelson, Cowie, Bolton & Britten (1955). It is particularly well suited to the characterization of trace amounts of radioactive substances, and its reliability compares with the method of isotope dilution.

EXPERIMENTAL

Materials and reagents

Nucleotides. The sodium salts of APP, APPP and APPPP were products of the Sigma Chemical Co., St Louis. The free acid of AP was obtained from the Pabst Laboratories, Milwaukee.

The pyridine salt of APP was prepared by mixing 15 mg. of the barium salt of APP (Schwarz Laboratories, Mount Vernon, N.Y.) with an aqueous suspension (approx. 1:1, v/v) of the pyridine salt of Dowex-50 (0·15 g. dry wt.). The barium salt dissolved on stirring, and the solution was separated on a microfilter. The resin was washed with 2 × 1 ml. of water, and the combined filtrate and washings were freeze-dried. The resulting powder was used as such.

Radioactive phosphate (*P**). Carrier-free ^{32}P-orthophosphate in dilute HCl was obtained from The Radiochemical Centre, Amersham, Bucks. It was evaporated to dryness in the reaction vessel by means of a stream of cold air, before other reagents were added. This procedure removes HCl, which interferes with the condensation reaction.

Radioactive pyrophosphate (*P*P**). This was prepared after the method of Bell (1950). To 0·1 mc of carrier-free P*, evaporated to dryness as described above, was added 0·05 ml. of 0·020 M-Na_2HPO_4 (1 μmole of Na_2HPO_4). The solution was again evaporated to dryness. The material was then heated at 290° for 24 hr.

After cooling it was dissolved in 0·05 ml. of water and chromatographed with solvent system 3 (see below). The paper was dried in a current of cold air. The exact locations of the spots on the chromatogram were best found by autoradiography. An exposure of 2 min. sufficed, owing to the high radioactivity of the material. The area of the paper containing the P*P* spot was cut out and eluted by the slow addition of water to the top edge of the suspended piece of paper. The first 0·2 ml. collected contained most of the radioactivity, yield 76%. The eluted P*P* was rechromatographed with solvent system 3, and was found to contain 99·4% of P*P* and 0·3% of P*. The material was eluted and rechromatographed a second time, and was found to contain 99·5% of P*P* and 0·1% of P*. After elution with water the twice-rechromatographed material was neutralized with pyridine.

NN'-Dicyclohexylthiourea. The preparation of this compound was based on the method of Skita & Rolfes (1920). *cyclo*Hexylamine (200 g., 2 moles) was mixed with 650 ml. of ethanol. Carbon disulphide (150 ml., 2·5 moles) was added slowly with stirring over a period of 30 min. The reaction mixture became very warm after about 1 min. Boiling was prevented by regulating the rate of addition of CS_2 and by adequate stirring. The mixture was cooled to 5°. The precipitate was filtered off, transferred to a 2 l. flask, and suspended in 1 l. of ethanol. The mixture was refluxed for 20 hr., during which time the precipitate dissolved and H_2S was evolved. The solution was cooled to 5°. The precipitate was filtered off, recrystallized from 2·5 l. of boiling methanol and dried *in vacuo* over anhydrous $CaCl_2$. Yield 145 g. (60%); m.p. 178–180°.

NN'-Dicyclohexylcarbodiimide (*DCC*). This was prepared by a modification of the method of Schmidt, Seefelder, Jennen, Striewsky & Martius (1951). Sodium hydroxide (120 g., 3 moles) was dissolved in 1 l. of water in a 2 l. round-bottomed flask. The solution was cooled to 0°, and 71 g. of Cl_2 (1 mole) was passed into the solution. This yielded a solution containing 1 mole of NaOCl, 1 mole of NaOH, and 1 mole of NaCl in 1 l. of water. Methylene chloride (225 ml.) was added, followed by 60 g. of *N,N'-dicyclo*hexylthiourea (0·25 mole). The mixture was stirred at room temp. for 5 hr., a reflux condenser being fitted to prevent the loss of methylene chloride. The methylene chloride layer was separated and was then stored at – 20° for 24 hr. Fine crystals of ice formed on the surface of the solution, and large crystals of sulphur formed on the bottom of the flask. The former were removed by filtration, the latter by decantation. (The dried sulphur crystals weighed 2·7 g.)

The methylene chloride was distilled off under reduced pressure. The residue was distilled from a 150 ml. flask immersed in a heated-oil bath. The first fraction of distillate, which included residual methylene chloride, was collected until the distillation temperature was 119° at 1 mm. Hg approx. It weighed 4·5 g. The main fraction was collected until the distillation temp. had risen to 125°. It consisted of the product, DCC, which was colourless and odourless, and weighed 33 g. The residue in the distillation flask (4·5 g.) was discarded. The product was redistilled. The main fraction weighed 27·5 g. Yield 53%.

Analytical methods

Chromatography. The *iso*propyl ether–90% formic acid solvent (3:2, v/v) of Krebs & Hems (1953) and Zetterström & Ljunggren (1951) was used for ascending chromatograms (solvent system 1). The *iso*butyric acid–N-NH_3 soln.–0·1 M ethylenediaminetetraacetic acid solvent (60:36:1, by vol.) of Krebs & Hems (1953) was used for descending chromatograms (solvent system 2). When these solvent systems were used consecutively according to the method of Krebs & Hems (1953), they are referred to as solvent system 1–2. After chromatography in solvent system 1, the paper carrying P and PP was cut off. This left insufficient paper to form a wick for chromatography of the nucleotides in solvent system 2. A piece of filter paper was therefore sewn on for this purpose with a toy sewing machine.

The *tert.*-butanol–90% formic acid–water solvent (80:5:20, by vol.) of Crowther (1954) was used for descending chromatograms (solvent system 3).

Potato apyrase. This enzyme catalyses the hydrolysis of the γ-phosphate of APPP at 0°, and the consecutive hydrolysis of the γ- and β-phosphates of APPP at 40° (Lee & Eiler, 1951; Thoai, Roche & An, 1954). The preparation used

for the work in this report was made and described by Whittam, Bartley & Weber (1955). It was used to determine the relative amounts of radioactivity in the γ and β positions of APPP.

Other methods. Phosphate-containing compounds on chromatograms were detected by the spraying method of Hanes & Isherwood (1949) or by autoradiography. Orthophosphate was estimated by the method of Berenblum & Chain (1938). Radioactive phosphorus was measured in a liquid counter (Veall, 1948). Ultraviolet photographs were made according to the method of Markham & Smith (1949). Autoradiographs of chromatograms were prepared with Kodak Blue Brand X-ray film. When the chromatograms contained labile compounds of low radioactivity the exposures were made at $-20°$.

PREPARATIONS AND RESULTS

Preparation of APP.* A 3 ml. glass-stoppered flask was used as reaction vessel. A volume (0·3 ml.) of carrier-free ^{32}P-orthophosphate solution, containing 1 mc/10 ml. was measured into the reaction flask and evaporated to dryness. Next, 10 mg. of AP and 0·50 ml. of pyridine–water (10:1, v/v) were added to the flask, followed by 0·20 g. of DCC. A glass bead was added, the flask was stoppered, and the stopper was secured by metal springs. The whole was shaken vigorously on a mechanical shaker. Complete solution occurred within 3 min. and a precipitate (probably di*cyclo*hexylurea) began to separate after about 1 hr. Shaking was stopped after 24 hr. Water (1 ml.) was added, and the mixture was shaken rapidly for 1 min. The flask was centrifuged slowly to separate the aqueous solution, which was then transferred to a clean, glass-stoppered tube by means of a drawn-out dropper. Free pyridine was removed by extracting the aqueous solution with 2×7 ml. of ether. A sample (5 μl.) of the aqueous extract was chromatographed on Whatman no. 1 paper with solvent system 1–2. Results of analyses performed on the chromatogram are shown in Table 1*a*. The remainder of the solution was applied to Whatman no. 3 MM paper as a band 20 cm. wide and was chromatographed with solvent system 1–2. The chromatogram was extracted by washing it in ether. The area corresponding to APP* was detected by autoradiography, and was

Table 1*a*. *Formation of APP**

The aqueous extract of the reaction products contained 1742000 counts/min., and the combined ether extracts, which were discarded, contained 148 000 counts/min. A volume (5 μl.) of the aqueous extract was cochromatographed with 0·2 μmole each of AP, APP and APPP, and 0·02 μmole each of P and PP, with solvent system 1–2. (For experimental details see text.)

Area on paper chromatogram	Radioactivity	
	Counts/min.	% of total
P	3150	21
PP	810	5
AP	570	4
APP	8460	57
APPP	1250	9
Rest of paper	520	4

Table 1*b*. *Identification and analysis of purified APP**

The solution of APP* contained 110000 counts/min. and 0·31 μg. of phosphorus/ml. A sample of the solution containing about 500 counts/min. was chromatographed in triplicate with solvent system 1–2. One sample (*A*) was run without additions. With the second sample (*B*) were cochromatographed 0·2 μmole each of P, AP, APP and APPP. With the third sample (*C*) was cochromatographed 0·5 μmole of APP. The irregularities of the APP* spot on the chromatogram of sample (*C*), which were revealed by autoradiography, coincided in every detail with those revealed by ultraviolet-light photography. A further sample (*D*) was heated with an equal volume of N-HCl at 100° for 10 min. before being chromatographed.

Area on paper chromatogram	Radioactivity (% of total)		
	Sample *A*	Sample *B*	Sample *D*
P	0	0	100
AP	0	0	0
APP	100	100	0
APPP	0	0	0
Rest of paper	0	0	0

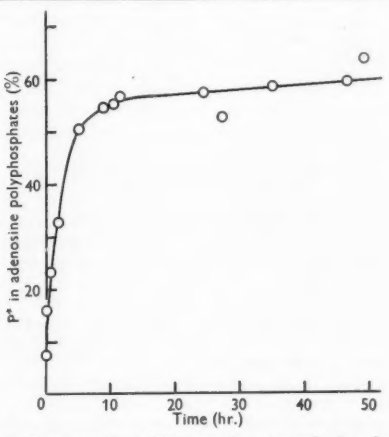

Fig. 1. Incorporation of P* into adenosine polyphosphates. The reaction mixture, which was shaken rapidly, contained 30 μc of carrier-free P*, 10 mg. of AP, and 0·2 g. of DCC in 0·5 ml. of pyridine–water (10:1, v/v). Samples (2·5 μl.) were withdrawn at the times indicated and placed on chromatograms. The latter were developed immediately with solvent system 1. The solvent front reached the start line about 3 min. after withdrawing the sample. (The reaction was found to continue on the paper if chromatography was not started immediately.) The developed chromatogram was analysed for radioactivity of adenosine polyphosphates and P.

cut out and eluted with ice-cold water. Samples of this solution were used for the chromatographic analyses shown in Table 1*b*. These show the solution to have contained essentially carrier-free APP*. The solution was stored frozen.

Similar reaction conditions were employed in a time-course experiment, the results of which are shown in Fig. 1.

Preparation of radioactive APPP (from APP plus P).* Carrier-free ^{32}P-orthophosphate solution (0·3 ml.) containing 1 mc/10 ml. was measured into a 3 ml. glass-stoppered reaction flask and evaporated to dryness. An amount (10 mg.) of the sodium salt of APP and 0·5 ml. of pyridine–water (10:1, v/v) were added to the flask, followed by 0·20 g. of DCC. A glass bead was added, the flask was stoppered, and the stopper secured by metal springs. The whole was shaken rapidly on a mechanical shaker for 6 hr. A small amount of solid remained in suspension. The aqueous extract of the reaction mixture was prepared and analysed as described for the preparation of APP*. Results are shown in Table 2*a*. The remainder of the aqueous extract was chromatographed on Whatman no. 3 MM paper with solvent system 1–2. The chromatogram was extracted by washing it in ether. The area corresponding to radioactive APPP was detected by autoradiography, and was cut out and eluted with ice-cold water. Samples of this solution were used for the chromatographic analyses shown in Table 2*b*. These show the solution to have contained radioactive APPP of high specific activity. An analysis of the distribution of P* in the β and γ positions of this compound by means of the action of potato apyrase is shown in Fig. 2. It shows two-thirds of the radioactivity in the γ-phosphate, and one-third in the β-phosphate of the APPP. The solution was stored frozen.

Similar results were obtained when the sodium salt of APP was replaced by the pyridine salt of APP.

*Preparation of radioactive APPP (from AP plus P*P*).* An aqueous solution of P*P*, neutralized with pyridine and containing $2{\cdot}29 \times 10^6$ counts/min., was evaporated to dryness in a 3 ml. glass-stoppered

Table 2*a*. *Formation of radioactive APPP (from APP plus P*)*

The aqueous extract of the reaction products contained 1 348 000 counts/min., and the combined ether extracts, which were discarded, contained 150 000 counts/min. A volume (5 μl.) of the aqueous extract of the reaction products was cochromatographed with 0·2 μmole each of AP, APP, APPP and APPPP, and 0·02 μmole each of P and PP, with solvent system 1–2. (For experimental details see text.)

Area on paper chromatogram	Radioactivity	
	Counts/min.	% of total
P	2820	24
PP	1140	10
AP	120	1
APP	1310	11
APPP	4270	36
APPPP	1010	9
Rest of paper	1170	10

Table 2*b*. *Identification and analysis of purified radioactive APPP*

The solution of APPP contained 132 400 counts/min. and 4·6 μg. of phosphorus/ml. A sample containing about 500 counts/min. was chromatographed in triplicate with solvent system 1–2. One sample (*A*) was run without additions. With the second sample (*B*) were cochromatographed 0·2 μmole each of AP, APP, APPP and APPPP, and 0·02 μmole each of P and PP. With the third sample (*C*) was cochromatographed 0·5 μmole of APPP. The irregularities of the radioactive APPP spot on the chromatogram of sample (*C*), which were revealed by autoradiography, coincided in every detail with those revealed by ultraviolet-light photography. A further sample (*D*) was heated with an equal volume of N-HCl at 100° for 10 min. before being chromatographed.

Area on paper chromatogram	Radioactivity (% of total)		
	Sample *A*	Sample *B*	Sample *D*
P	3	0	100
PP	0	0	0
AP	0	0	0
APP	7	6	0
APPP	89	93	0
APPPP	1	1	0

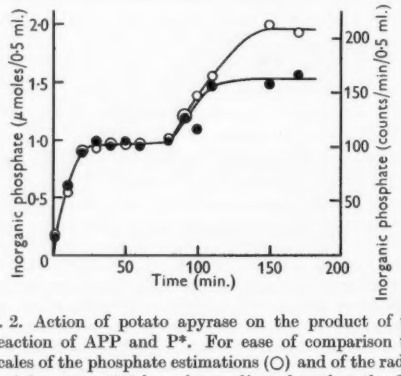

Fig. 2. Action of potato apyrase on the product of the reaction of APP and P*. For ease of comparison the scales of the phosphate estimations (○) and of the radioactivity assays (●) have been adjusted so that the first plateau of each curve coincides. The incubation mixture consisted of 0·2 ml. of M sodium succinate, pH 6·8; 0·2 ml. of 0·1 M-$CaCl_2$; 0·6 ml. of 0·01 M APPP; 0·1 ml. of a solution of the reaction product containing 1000 counts/min.; 0·2 ml. of enzyme (0·2 mg. of protein); and 0·9 ml. of water. Temp., 0° during first 80 min., thereafter 38°. Samples (0·2 ml.) were withdrawn for analysis at the times indicated, and pipetted into 3 ml. of ice-cold trichloroacetic acid (5%, w/v).

reaction flask. Next, 10 mg. of AP and 0·20 g. of DCC were added, followed by 0·5 ml. of pyridine–water (10:1, v/v), and a glass bead. The whole was shaken rapidly for 6 hr. An aqueous extract of the reaction mixture was prepared and analysed as described for the preparation of APP*. Results are shown in Table 3*a*. The remainder of the aqueous extract was chromatographed on Whatman no. 3 MM paper with solvent system 1–2. A slight modification of the chromatographic method was adopted. After chromatography with solvent system 1, the paper was dried in a current of cold air, and an autoradiograph was prepared. The latter was used as a guide for cutting the chromatogram between the APP*P* and P*P* areas. Chromatography with solvent system 2 was then carried out as described previously. The area corresponding to APP*P* was detected by autoradiography and was cut out and eluted with ice-cold water. Samples of this solution were used for the chromatographic analyses shown in Table 3*b*. These show the solution to have contained radioactive APPP of very high specific activity. An analysis of the distribution of P* in the β and γ positions of this compound by means of the action of potato apyrase is shown in Fig. 3. It shows that the radioactivity was equally divided between the β- and γ-phosphates of the APPP. The solution was stored frozen.

Table 3*a*. *Formation of radioactive APPP (from AP plus P*P**)

The aqueous extract of the reaction products contained 1 750 000 counts/min., and the combined ether extracts, which were discarded, contained 338 000 counts/min. A volume (5 μl.) of the aqueous extract was cochromatographed with 0·2 μmole each of AP, APP, APPP and APPPP, and 0·02 μmole each of P and PP, with solvent system 1–2. (For experimental details see text.)

Area on paper chromatogram	Radioactivity	
	Counts/min.	% of total
P	380	3
PP	5660	39
AP	110	1
APP	1320	9
APPP	5750	39
APPPP	570	4
Rest of paper	820	6

Table 3*b*. *Identification and analysis of purified APP*P**

The solution of APP*P* contained 586 500 counts/min. and 5·5 μg. of phosphorus/ml. A sample containing about 5000 counts/min. was chromatographed in duplicate with solvent system 1–2. One sample (*A*) was run without additions. With the second sample (*B*) were cochromatographed 0·2 μmole each of AP, APP, APPP and APPPP, and 0·02 μmole each of P and PP. A third sample (*C*) was cochromatographed with 0·5 μmole of APPP with solvent system 1–2. The irregular outline of the APP*P* spot on the chromatogram of sample (*C*), which was revealed by autoradiography, coincided in every detail with that revealed by ultraviolet-light photography. A further sample (*D*) was heated with an equal volume of N-HCl, at 100° for 15 min. before being chromatographed.

Area on paper chromatogram	Radioactivity (% of total)		
	Sample *A*	Sample *B*	Sample *D*
P	1	1	100
PP	1	2	—
AP	0	1	—
APP	6	7	—
APPP	88	82	—
APPPP	3	4	—
Rest of paper	3	3	0

Preparation of radioactive APPPP. Carrier-free ^{32}P-orthophosphate solution (0·03 ml.) containing 1 mc/ml. was measured into a 3 ml. glass-stoppered reaction flask and evaporated to dryness. Next, 9 mg. of the sodium salt of APPP and 0·5 ml. of pyridine–water (10:1, v/v) was added to the flask, followed by 0·20 g. of DCC. A glass bead was added, the flask was stoppered securely, and shaken rapidly for 6 hr. The aqueous extract of the reaction mixture was prepared and analysed as described for the preparation of APP*. Results are shown in Table 4*a*. The remainder of the aqueous extract was chromatographed on Whatman no. 3 MM paper with solvent system 1–2. The chromatogram was extracted by washing it in ether. The area on the chromatogram corresponding to radioactive APPPP was detected by autoradiography, and was cut out and eluted with ice-cold water. Samples of this solution were used for the chromatographic

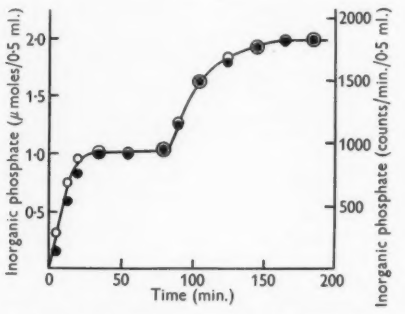

Fig. 3. Action of potato apyrase on the product of the reaction of AP and P*P*. For ease of comparison the scales of the phosphate estimations (○) and the radioactivity assays (●) have been adjusted so that the first plateau of each curve coincides. The test solution and conditions were similar to those described in Fig. 2, except for the solution of the reaction product, of which 0·1 ml. of a solution containing 11 500 counts/min. was added.

analyses shown in Table 4*b*. These show the solution to have contained mainly radioactive APPPP. The solution was stored frozen.

DISCUSSION

The methods of preparing labelled adenosine polyphosphates which are described in this paper are simple and quick, and the yields in terms of P* used are high.

Table 4*a*. *Formation of radioactive APPPP*

The aqueous extract of the reaction products contained 3 700 000 counts/min., and the combined ether extracts, which were discarded, contained 612 000 counts/min. A volume (5 μl.) of the aqueous extract of the reaction products was cochromatographed with 0·2 μmole each of AP, APP, APPP, and APPPP and 0·02 μmole each of P and PP, with solvent system 1–2. (For experimental details see text.)

Area on paper chromatogram	Radioactivity	
	Counts/min.	% of total
P	18 060	66
PP	2 210	8
AP	20	0
APP	340	1
APPP	1 640	6
APPPP	3 070	11
Rest of paper	1 980†	7

† Autoradiography showed this radioactivity to be mainly concentrated in an area just above APPPP.

Table 4*b*. *Identification and analysis of purified radioactive APPPP*

The solution of radioactive APPPP contained 114 600 counts/min. and 31 μg. of phosphorus/ml. A sample containing about 500 counts/min. was chromatographed in duplicate with solvent system 1–2. One sample (*A*) was run without additions. With the second sample (*B*) were cochromatographed 0·2 μmole each of AP, APP, APPP and APPPP, and 0·02 μmole each of P and PP. A third sample (*C*) was cochromatographed with 0·5 μmole of APPPP with solvent system 2. The irregular outline of the radioactive APPPP spot on the chromatogram of sample (*C*), which was revealed by autoradiography, coincided in every detail with that revealed by ultraviolet-light photography. A further sample (*D*) was heated with an equal volume of N-HCl, at 95° for 5 min., before it was chromatographed with solvent system 1–2.

Area on paper chromatogram	Radioactivity (% of total)		
	Sample *A*	Sample *B*	Sample *D*
P	2	2	82
PP	0	2	11
AP	0	0	0
APP	0	2	1
APPP	8	12	1
APPPP	84	76	2
Rest of paper	6	7	3

The preparation of APP* has presented the greatest practical difficulties in the past. It was obtained as a by-product in the preparation of APP*P*, or it was prepared by the selective enzymic cleavage of APP*P*, which had in turn to be prepared enzymically (e.g. Webster & Varner, 1954; Lieberman, 1956). With AP and P* as starting materials, the method which is reported in this paper yields APP* of very high specific activity, in high yield in terms of P* added. The time-course of the reaction for the experimental conditions which were employed is shown in Fig. 1. The rate of condensation of P* with AP is rapid initially, but it decreases sharply after about 6 hr. The data of Table 1*b* demonstrate that the radioactivity of the product is found entirely in the orthophosphate released by hydrolysis in 0·5 N-HCl at 100° for 10 min. The ribose 5-phosphate ester linkage is not hydrolysed under these conditions (Eggleston, 1954). Since there is no radioactivity in the AP or in ribose 5-phosphate, this shows clearly that the radioactivity is confined to the β-phosphate of APP.

When the starting materials are APP and P*, the main radioactive products are a mixture of APPP* and APP*P*. In the experiment described above these were formed in equal amounts, as is indicated by the γ- to β-labelling ratio of 2:1 (see Fig. 2). The manner in which the β-phosphate becomes labelled is probably as follows. During the reaction some breakdown to AP occurs, and some APP* is formed. The latter then gives rise to APP*P*. The labelling of the β position can probably be reduced, if not abolished, by varying the reaction conditions; for example, by using a shorter reaction time. Similar considerations apply when the starting materials are APPP and P* and the main radioactive product is APPPP. Here, too, some labelling of the γ position, and possibly also of the β position, is to be expected. That some P is released when APP or APPP are used as starting materials is indicated by the lower specific activities of the products obtained from these compounds.

When the starting materials are AP and P*P*, the method yields APP*P* of high specific activity, in high yields in terms of P*P* added. The APP*P* so formed was labelled in the γ and β positions in the ratio of 1:1, as is to be expected (see Fig. 3). This is the first successful use of PP in the preparation of polyphosphates with DCC as condensing agent.

The formation of symmetrical dinucleotides such as, for example, APPA was not observed during these preparations. A possible explanation is that in the solvents used, the symmetrical dinucleotides have R_F values similar to those of the corresponding mononucleotides from which they would be expected to arise, and that they thus escape detection.

The method is capable of extension to the preparation of other nucleoside polyphosphates labelled with radioactive phosphorus.

SUMMARY

1. The reaction of orthophosphate with adenosine 5′-monophosphate, diphosphate and triphosphate in the presence of *NN′*-di*cyclo*hexylcarbodiimide has been used for the preparation of labelled adenosine polyphosphates.

2. The adenosine diphosphate so prepared is labelled exclusively in the terminal phosphate group. The adenosine triphosphate and adenosine tetraphosphate so prepared are labelled in the terminal phosphate group, and to a lesser extent in the penultimate phosphate group.

3. A feature of the method is that carrier-free labelled orthophosphate can be used without prior dilution with unlabelled orthophosphate.

4. The reaction of pyrophosphate with adenosine 5′-monophosphate in the presence of *NN′*-di*cyclo*hexylcarbodiimide has been used for the preparation of labelled adenosine triphosphate.

5. The adenosine triphosphate so prepared is labelled in the β- and γ-phosphate groups to an equal extent.

6. An improved method for the preparation of *NN′*-di*cyclo*hexylcarbodiimide is described.

I am indebted to Professor H. A. Krebs for the privilege and pleasure of working in his laboratory. I wish to thank Mr R. Hems for discussions and much helpful advice, Mr K. Jones for advice on the use of ultraviolet photography, Dr H. L. Kornberg for advice on autoradiography, Dr W. Bartley for a gift of potato apyrase and Professor J. Baddiley for pointing out to me the necessity of the refluxing procedure in the preparation of *N,N′*-di*cyclo*hexylthiourea.

My thanks go to the Sigma Chemical Co., St Louis, Missouri, for a most generous gift of adenosine tetraphosphate.

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Properties of Limpet β-Glucuronidase

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The common limpet (*Patella vulgata*) was found to contain an α-glucuronidase (Conchie, Levvy & Marsh, 1956). This mollusc, like many others, was already known to possess β-glucuronidase activity (Dodgson, Lewis & Spencer, 1953), and may be one of the best sources of this enzyme for use in the hydrolysis of steroid glucuronides in urine. It was necessary to compare the properties of the two enzymes, and the present paper is concerned with the β-glucuronidase. Most of the experiments were done with phenolphthalein β-glucuronide, which has come to be the standard substrate for this enzyme. The opportunity was also taken to examine the fluorimetric method of Mead, Smith & Williams (1955), 4-methylumbelliferone β-glucuronide being used as substrate. Among other possible advantages is the fact that this substrate can be synthesized (Marsh & Levvy, 1956*a*).

EXPERIMENTAL

Preparation of the enzyme. Freshly dissected limpet visceral humps were suspended in water (10 ml./g. of moist tissue) by means of a glass homogenizer. According to Dodgson *et al.* (1953), most of the β-glucuronidase activity is extracted by homogenizing in water. The homogenate was frozen and thawed, and centrifuged at 10000 *g* for 15 min. The supernatant was fractionated with $(NH_4)_2SO_4$ at pH 5·2, with centrifuging as before, and the fraction separating between 20 and 80% saturation was retained and dissolved in water (2·5 ml./g. of tissue). This preparation kept indefinitely at −20° or 0°. There was slow separation of traces of insoluble material which could be removed without loss in activity. The stock preparation was usually diluted 400-fold before use (final concn. in terms of moist tissue, 0·1%); at this dilution, the activity fell about 10% daily at 0°.

Enzyme assay with phenolphthalein glucuronide. The assay with phenolphthalein glucuronide (Talalay, Fishman & Huggins, 1946) was done by the general procedure described previously (Levvy, 1952). The volume of the incubation mixture was 4 ml., and after 1 hr. at 37° the reaction was stopped and the phenolphthalein colour developed by the addition of 4 ml. of glycine–Na_2CO_3 buffer. In the pH–activity experiments, NaOH was then added to bring all the tubes to the same pH, and the final volume was made up to 10 ml. instead of the customary 8 ml. All results were read from the appropriate calibration curve.

Except where otherwise stated, the acetate buffer used in the experiments was made with NaOH. The phthalate buffer contained the potassium salt.

Fluorimetric measurements

The following modifications were made in the method of Mead *et al.* (1955).

Instrument. An H764 Spekker fluorimeter (with photomultiplier) was used. The 8 ml. cells contained exactly 6·5 ml. of solution and were used without covers. The primary filters were Wood's glass and the secondary filter was Wratten 47: the other types and combinations of filter which were tried were no better, and most of them were less satisfactory.

Glycine buffer. To develop the fluorescence in 4-methylumbelliferone solutions, 0·2 M glycine–NaOH buffer, pH 10·3, was employed. This had a blank somewhat higher than water, but the same as equimolar A.R. KCl solution. It was important to use A.R. glycine, since less pure material had a very high blank. The pure buffer gave a transmission of 33% (equivalent to 0·0008 μg. of 4-methylumbelliferone/ml.) when read against a quinine bisulphate standard containing 0·05 μg./ml., and this blank determined the lower limit of the method with our instrument.

Calibration curve for 4-methylumbelliferone. On exposure to sunlight or to an ultraviolet lamp, 4-methylumbelliferone solutions (at least up to 0·8 μg./ml.) were found to be unstable at neutral pH, and very unstable in the glycine buffer. At pH 4–5, the solutions were much more stable, but there was considerable decomposition in 0·05 M trichloroacetic acid, even in the dark. Attempts to stabilize the solutions in glycine buffer by adding ascorbic acid or sodium dithionite were unsuccessful.

Dilute solutions of 4-methylumbelliferone were prepared just before use and kept in the dark as much as possible. To 5 ml. of solution was added 5 ml. of glycine buffer, and the fluorescence was measured at once against a suitable quinine bisulphate standard in 0·1 N-H_2SO_4. Linear calibration curves were obtained with quinine bisulphate standards ranging from 0·05 μg./ml. (for 0·0002–0·0015 μg. of 4-methylumbelliferone/ml.) to 2 μg./ml. (for 0·01–0·075 μg. of 4-methylumbelliferone/ml.).

RESULTS

Variation in activity with pH. Dodgson *et al.* (1953) observed a broad optimum at pH 4 for the hydrolysis of 0·045 M *p*-chlorophenyl glucuronide in 0·5 M acetate buffer by limpet β-glucuronidase. As shown in Fig. 1, the optimum for the hydrolysis of 0·00125 M phenolphthalein glucuronide in 0·05 M acetate buffer made with NaOH was at pH 3·8; replacement of NaOH by KOH had no effect on the curve. The position of the peak could be altered, without marked change in the optimum activity, by varying the concentration of buffer or substrate. Changes in pH optimum have been noted previously with β-glucuronidase from other sources (Smith & Mills, 1953; Levvy, 1954).

Fig. 1 also shows the pH–activity curve for the hydrolysis of 0·00125 M phenolphthalein glucuronide in 0·25 M phthalate buffer: the peak was at pH 4·6. The shape of the curve, as well as the position of the

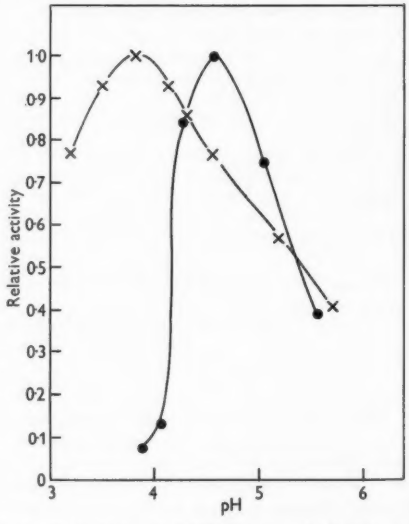

Fig. 1. Hydrolysis of 0·00125 M phenolphthalein glucuronide by limpet β-glucuronidase at varying pH in 0·05 M acetate (×) and 0·25 M phthalate buffer (●). (The optimum activity with each buffer is taken as 1·0.)

peak, could be altered by changing the buffer concentration. Optimum activity was only about 10% higher in phthalate than in equimolar acetate buffer, allowing for the effect of the buffer on the phenolphthalein colour. Smith & Mills (1953) noted considerable enhancement of animal β-glucuronidase activity by phthalate as compared with acetate buffer.

Variation in stability with pH *and temperature.* Measured samples of an enzyme preparation, initially at pH 6–7, were adjusted to the required pH with HCl or NaOH at 0°. After a predetermined period at 0° or 37°, the pH was brought back at 0° to the original figure, and the activities were compared with that of a control kept at 0°. Assays were done with 0·00125 M phenolphthalein glucuronide in 0·05 M acetate buffer, pH 4·14. In agreement with Dodgson *et al.* (1953), the enzyme was found to be rapidly inactivated below pH 3 at 0° (Fig. 2). At 37° it was slowly inactivated below pH 4. On the other hand, it displayed considerable stability at alkaline pH values. For purposes of comparison, experiments were also done with preparations of mouse-liver β-glucuronidase (Levvy, 1952), which showed a considerably narrower range of stability at varying pH. Rumen β-glucuronidase was stable only at pH 6–7 (Karunairatnam & Levvy, 1951). On the basis of the present experiments, a pH of 4·1–4·2 was adopted for routine assay of limpet β-glucuronidase.

Heating an unbuffered limpet preparation (approx. pH 5) for 5 min. at the usual dilution taken for assay caused 15% inactivation at 60° and 35% at 70°.

Effect of varying concentrations of substrate and inhibitors. Fig. 3 shows the variation in enzyme activity with the phenolphthalein glucuronide concentration, and the effects of saccharo-1:4-lactone and boiled mucic acid solution (Levvy, 1952), both of which acted as competitive inhibitors. When the results were analysed by the method of Lineweaver & Burk (1934), this particular experiment gave values of $2{\cdot}7 \times 10^{-5}$ M for K_m, the dissociation constant of the enzyme–substrate complex, and $0{\cdot}89 \times 10^{-7}$ M and $9{\cdot}8 \times 10^{-6}$ M for K_i, the dissociation constant of the enzyme–inhibitor complex, for saccharo-1:4-lactone and boiled mucic acid respectively. Mean values for the dissociation constants obtained in a number of experiments were: phenolphthalein glucuronide, $2{\cdot}4 \times 10^{-5}$ M; saccharo-1:4-lactone, $1{\cdot}05 \times 10^{-7}$ M; boiled mucic acid, $6{\cdot}7 \times 10^{-6}$ M. The affinities of the first two compounds were thus five times greater than those with mouse-liver β-glucuronidase (Levvy, 1952), but the relative affinities were the same (affinity = 1/dissociation constant). Boiled mucic acid had a somewhat higher relative affinity for the limpet than

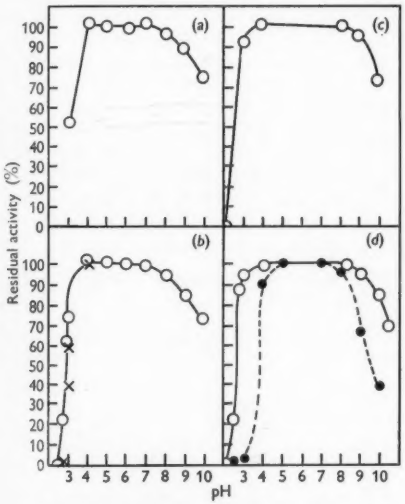

Fig. 2. Stability of limpet and mouse-liver β-glucuronidase at varying pH: (*a*) 1 hr. at 37°; (*b*) 15 min. at 37°; (*c*) 1 hr. at 0°; (*d*) 1 min. at 0°. ○, Limpet enzyme adjusted with NaOH and HCl; ×, limpet enzyme adjusted with NaOH and acetic acid; – –●– –, mouse-liver enzyme adjusted with NaOH and HCl.

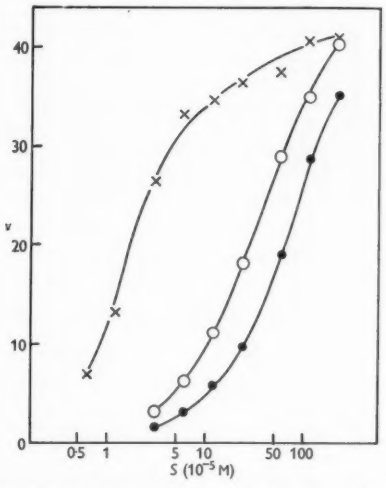

Fig. 3. Effect of varying the substrate concentration (*S*) on the rate of hydrolysis (*v*) of phenolphthalein glucuronide by limpet β-glucuronidase in the absence and the presence of inhibitors: ×, no inhibitor; ○, $1{\cdot}25 \times 10^{-4}$ M mucic acid (boiled); ●, $2{\cdot}5 \times 10^{-6}$ M saccharo-1:4-lactone. Acetate buffer, pH 4·14, final concn. 0·05 M.

for the mouse-liver enzyme: in this instance the term affinity has no theoretical significance since inhibition is probably due to galactosaccharolactone present in the boiled solution.

Similar experiments were done in which the fluorimetric substrate, 4-methylumbelliferone glucuronide (Mead *et al.* 1955), prepared by chemical synthesis (Marsh & Levvy, 1956*a*), was treated as a competing substrate in the hydrolysis of phenolphthalein glucuronide, and a value of $1{\cdot}75 \times 10^{-5}$M was obtained for K_i. Control experiments showed that fluorescence due to liberated 4-methylumbelliferone did not pass the filter in the phenolphthalein estimation.

Inhibition by excess of phenolphthalein glucuronide was not seen with limpet β-glucuronidase. Maximal velocity of hydrolysis was reached with this substrate at a concentration of 0·00125M.

Effects of varying the period of hydrolysis and the enzyme concentration. The rate of hydrolysis of 0·00125M phenolphthalein glucuronide in 0·05M acetate buffer, pH 4·14, remained unchanged during incubation for 4 hr. at 37°. Increasing the concentration of the enzyme preparation during incubation for 1 hr. under the same conditions caused a slight net increase in activity per unit of enzyme (Fig. 4). Effects of the same order (10%) could be produced by addition of a filtrate from a boiled, relatively concentrated preparation to the usual concentration of enzyme (1 'unit'). A diffusate from an unboiled preparation did not produce this effect.

Hydrolysis of 4-methylumbelliferone β-glucuronide. Fig. 5 shows that the peak in the pH–activity curve obtained for the hydrolysis of 10^{-5}M 4-methylumbelliferone glucuronide (synthetic) by limpet β-glucuronidase in 0·05M acetate buffer was at pH 3·8. The stock enzyme preparation had to be diluted 80 000-fold before use (corresponding to a final tissue concentration of 0·0005%), and 0·5 ml. of this diluted enzyme was employed in a final incubation volume of 4 ml. Incubation was carried out for 1 hr. at 37° in the dark, and 5 ml. of 0·2M glycine buffer was added. All tubes were brought to the same final pH by adding a predetermined volume of NaOH, and the final volume was brought to 10 ml. The tubes were stored in the dark at 0° until read against a quinine bisulphate standard containing 0·2 μg./ml. At the concentration of substrate employed, the enzyme was by no means saturated, the dissociation constant determined indirectly having a value of $1{\cdot}75 \times 10^{-5}$M (see above), but the maximum hydrolysis was only 0·2%.

There was measurable fluorescence in the 10^{-5}M substrate control, corresponding to a figure of 0·001–0·003 μg. of 4-methylumbelliferone/ml. after adding glycine buffer. This value places an upper limit on the substrate concentration that may be employed, depending on the strength of the quinine standard, and thus introduces difficulties into more detailed kinetic studies. The substrate was freed

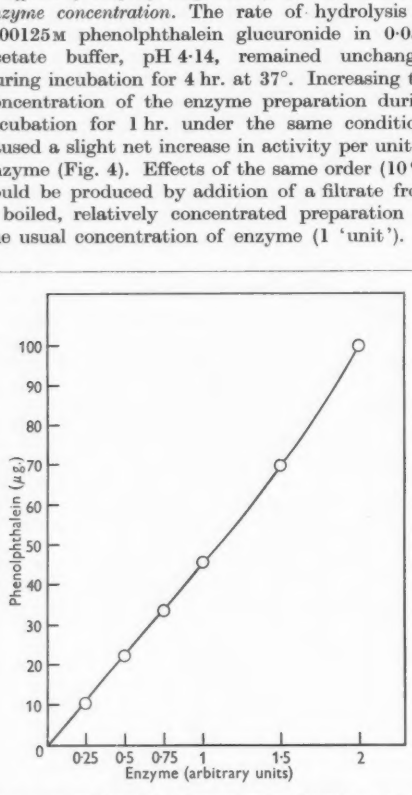

Fig. 4. Phenolphthalein liberated from 0·00125M phenolphthalein glucuronide in 0·05M acetate buffer, pH 4·14, by varying amounts of limpet β-glucuronidase after 1 hr. at 37°.

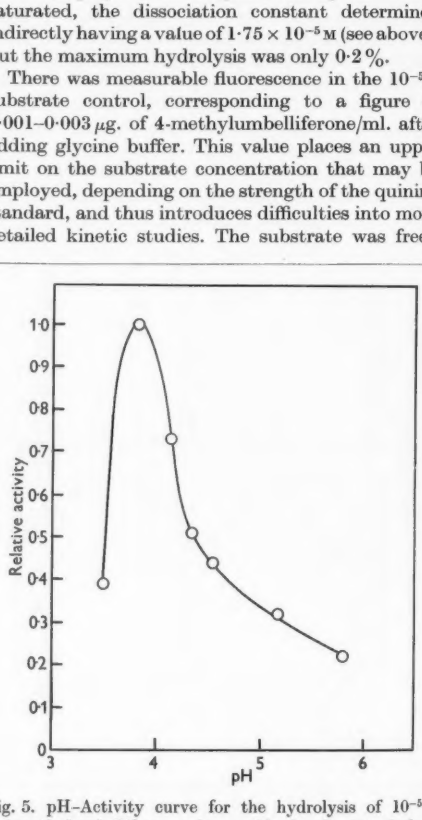

Fig. 5. pH–Activity curve for the hydrolysis of 10^{-5}M 4-methylumbelliferone glucuronide by limpet β-glucuronidase in 0·05M acetate buffer.

from last traces of 4-methylumbelliferone before use by precipitation from ethanol solution with ether.

Effects of metal ions. Doyle, Katzman & Doisy (1955) have described the inhibition of the β-glucuronidase from *Escherichia coli* by Cu^{2+}, Ag^{+} and Hg^{2+} ions in low concentration, and the reversal of inhibition by chelating agents and by L-cysteine. As shown in Table 1, limpet β-glucuronidase was inhibited by Ag^{+} and Hg^{2+} ions, but only very slightly by Cu^{2+} ions. Inhibition by both Ag^{+} and Hg^{2+} ions was reversed by ethylenediaminetetraacetic acid (EDTA) and by L-cysteine, the latter being much more efficient in overcoming inhibition. The action of silver could also be overcome very efficiently by adding Cl^{-} ions. No other metal ion tested (Zn^{2+}, Co^{2+}, Ba^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}) was any more effective than Cu^{2+} ions, nor were sodium arsenite and sodium borate. Unlike the *E. coli* preparation, which is activated by EDTA and L-cysteine in absence of added metal ions (Doyle *et al.* 1955), limpet β-glucuronidase was unaffected by EDTA alone and was slightly inhibited by L-cysteine.

Identification of free glucuronic acid in the enzyme hydrolysate. This was done chromatographically as described in an earlier paper (Marsh & Levvy, 1956*b*), and the only spots obtained were those for D-glucurone and D-glucuronic acid. There was no trace of pentose.

For the enzymic hydrolysis, 5 ml. of conc. stock limpet enzyme was incubated with 5 ml. of 0·02 M phenolphthalein glucuronide, pH 4·1, for 4 hr. at 37°. The mixture was then dialysed against 100 ml. of water for 72 hr. at 0°, and the diffusate, after adjustment to pH 2·5 with H_2SO_4, was extracted in a separating funnel with ethyl acetate until free from phenolphthalein and unhydrolysed glucuronide. Glucuronic acid was determined in a portion by measuring the reducing power (Levvy, 1946), and the total corresponded to 17·4 mg. (90% hydrolysis of the substrate), after correction for the enzyme blank. Substrate incubated alone showed no liberation of reducing substance or phenolphthalein at this stage.

The aqueous solution was shaken with $BaCO_3$ to remove H_2SO_4, filtered, concentrated *in vacuo* to 20 ml., and treated with Amberlite IR-120 (H) for 1 hr. The filtrate was maintained at 100° for 15 min. and evaporated to dryness *in vacuo*, and the resulting gum was dissolved in water for chromatography. As chromatographic control, a solution of sodium glucuronate of similar concentration was treated likewise after deionization.

DISCUSSION

The main object of this work was to lay a foundation for distinguishing between limpet β-glucuronidase and the other hexuronidases present in the preparation, namely, α-glucuronidase and α- and β-galacturonidase (Conchie *et al.* 1956), as well as for purifying the β-glucuronidase. From our present results, the limpet visceral hump is exceptionally high in β-glucuronidase activity in comparison with most animal tissues (cf. Fishman, 1955), giving a figure of approximately 100 000 phenolphthalein units/g. wet wt. Billet (1954) found that most of the β-glucuronidase activity in the Roman snail (*Helix pomatia*) was confined to the digestive gland.

For most purposes, the fluorimetric method for β-glucuronidase assay (Mead *et al.* 1955) is probably too exacting to displace the phenolphthalein method (Talalay *et al.* 1946). The fluorimetric method may, however, be useful when extreme sensitivity is required as, for example, in the assay of normal body fluids. By the use of the Spekker absorptiometer and the H 764 Spekker fluorimeter (quinine bisulphate standard 0·2 μg./ml.), the difference in sensitivity between the two methods was about

Table 1. *Effects of copper sulphate, silver nitrate and mercuric chloride on limpet β-glucuronidase activity, and the reversing actions of EDTA and L-cysteine*

Residual activity is expressed as percentage of control. Substrate: 0·00063 M phenolphthalein glucuronide. Buffer: 0·05 M acetate, pH 4·14.

		Reversing agent				
			EDTA		Cysteine	
Metal ion	Final concn. (M)	None	0·002 M	0·0002 M	0·002 M	0·0002 M
None	—	100	100	100	75	95
Cu^{2+}	0·001	88	—	—	—	—
Ag^{+}	0·001	0	—	—	—	—
	0·0001	26	43	35	84	100
	0·000025	84	—	88	—	—
Hg^{2+}	0·001	0	—	—	—	—
	0·0001	10	—	26	—	47
	0·000025	18	41	38	70	69

1000-fold; this was reduced to 100-fold, in terms of total aglycone released, when the micro-scale modification (Levvy, 1954) of the phenolphthalein method was employed. The greater sensitivity of the fluorimetric method was due almost entirely to the greater sensitivity of fluorimetric as compared with colorimetric measurements, since the rates of hydrolysis of 4-methylumbelliferone glucuronide and phenolphthalein glucuronide by a limpet preparation were much the same. Since many of the difficulties associated with the fluorimetric method were due to the instability of the aglycone at alkaline pH values, the fluorimetric assay might be improved by seeking a new substrate of suitable structure.

SUMMARY

1. Some of the properties of β-glucuronidase from *Patella vulgata* have been studied, including its action on phenolphthalein glucuronide and 4-methylumbelliferone glucuronide, and the effects of various inhibitors.

2. In dilute acetate buffer the pH optimum for both substrates was at pH 3·8.

3. The following dissociation constants (at 37°) were obtained for complexes formed by the enzyme: phenolphthalein glucuronide, $2{\cdot}4 \times 10^{-5}$ M; 4-methylumbelliferone glucuronide, $1{\cdot}75 \times 10^{-5}$ M; saccharo-1:4-lactone, $1{\cdot}05 \times 10^{-7}$ M; boiled mucic acid solution, $6{\cdot}7 \times 10^{-6}$ M.

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